In planta expression of exocellulase enzymes for Bio-ethanol production

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Parsu Ram Budathoki
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List of Abbrevation

AFM - Atomic force microscopy

5’ - Upstream of the sequence of interest

3’ - Downstream of sequence of interest

% - Per cent

°C - Degree Celsius

bp - Base pair

BSA - Bovine serum albumin

CaMV - Cauliflower mosaic virus

CBHI - Cellobiohydrolase

CIB - Chloroplast Isolation Buffer

CD - Catalytic Domain

CBM - Carbohydrate-binding module

E1 - endoglucanase

EG - Endoglucanase

BG - β-glucosidase

SLH - Surface layer homology domain

EDTA - Ethylenediaminetetraacetic acid

cDNA - Complementary DNA

CTAB - Cetyl Trimethyl Ammonium Bromide

H_2O - distilled water

DNA - Deoxyribonucleic acid

DGDG - diagalactosyldiacylglycerol

dNTPs - Deoxyribonucleotide triphosphate

DTT - Dithiothreitol

GH Glycosyl Hydrolase

GUS β - glucuronidase

H - hour

Kb - Kilobase

kDa - Kilodalton

LB - Luria-Bertani media

LPS - Lipopolysaccharides

µg - Microgram

µl - Microlitre

µM - Micromolar

min - Minute

MGDG - monogalactosyldiacylglycerol

mg - Milligram

ml - Millilitre

mM- Millimolar

M - Molar

MSO - Murashige and Skoog salt

MUC - 4 methyl-Umbelliferyl –β-D-cellobiopyranoside

NOS - nopaline synthase

ng - Nanogram

OD - Optical density

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate buffer saline

PCR - Polymerase chain reaction

pDNA - Plasmid DNA

ptDNA - plant DNA

PMSF - Phenyl-methylsulfonyl fluoride

rpm- Round per minute

RT-PCR Reverse transcription

s - Second

SDS - Sodium dodecyl sulphate

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

T - DNA transfer DNA

TLP - total leaf protein

TSP - total soluble protein

TEM - Transmission electron microscopy

U - Unit

UV - Ultra violet

WT - Wild-type

v/v - Volume per volume

w/v - Weight per volume

Abstract

The use of bioethanol as an alternative fuel can help reduce greenhouse emissions associated with the consumption of crude petroleum oil and also slow the depletion of fossil fuel reserves. However, first generation bioethanol produced from starch or sugar crops cannot meet the enormous demand for fuel ethanol due to limited production capacity using available agricultural land and the ensuing competition for food and feed production.

The production of ethanol from lignocellulosic crop biomass is challenging, but considering the availability of abundant quantities available globally, it has the potential to help meet the increasing requirement for clean and sustainable energy generation in the future.

One of the major challenges in making cellulosic ethanol cost-competitive is to reduce the production of cellbiohydrolase (CBH) the predominant enzyme required for the degradation of biomass into simple sugars prior to fermentation.

This project evaluated the expression of CBHI in the chloroplasts of tobacco, this was based on the premise that chloroplast based expression of other recombinant proteins have shown increased yield.Two genes encoding exocellulases; *cbhI* from *Trichoderma reesei,* and a novel gene *CO14* (a protozoan cellulase isolated from termite gut) were selected for expression. There are no reports of these enzymes having been expressed in chloroplasts.

This project generated transgenic tobacco lines that produced very limited amounts of recombinant exocellulases (CBHI/CO14) in the chloroplasts, however this expression generated a phenotype in that had major changes in the choloroplast

ultrastructure,; indicating that in plants the chloroplast is not a suitable organelle for transgenic expression of exocellulases.

Chapter 1: Project background

The burning of fossil fuels results in greenhouse gas emissions contributing to global warming and climate change. The burning of biofuels can improve the greenhouse emission-balance that comes from fossil fuels use and help slow down the depletion of fossil fuel reserves. The use of biofuels as a part of the fuel mix for transportation engines may provide cheap, clean, reliable energy sources that could contribute to future energy demands in a sustainable manner (Atabani *et al.,* 2012, Tollefson, 2010, Demirbas, 2007). However, ethanol produced from starch or sugar crops cannot meet the enormous demand for fuel ethanol due to the limited production capacity using available agricultural land and the competition for food and feed production (Sainz, 2011). The production of ethanol from lignocellulosic biomass is challenging, but considering the availability of abundant quantities of biomass globally, it has the potential to meet the increasing requirements around clean energy generation in the future (Sticklen, 2006, Kim and Dale, 2004). The main challenge in commercialisation of cellulosic ethanol is the cost of cellulases, particularly the production cost of cellobiohydrolases (CBHs) which are the dominant enzymes required for hydrolysis of cellulose but their production via microbial fermentation is inefficient and thereby increases the cost. The main aims of this project were; to generate transplastomic tobacco lines expressing functionally active exocellulases (CBHI/CO14) and to compare the efficiency of expression of the two enzymes after integration at trnV/rps7/12 locus within the tobacco plastid genome.

1.1 History of cellulosic ethanol production

In 1819 the French chemist Henri Braconnot discovered that cellulose could be hydrolysed into fermentable sugar by using sulphuric acid (Rudolf *et al.,* 2009, Singh, 2013). In 1838 French chemist Anselma Payen was able to isolate and purify cellulose from biomass (Singh, 2013). In 1898 the first commercial ethanol production began in Germany by a process using acid hydrolysis of cellulose which produced about 7.6 litres of ethanol per 100 kg of wood chips per tonne of biomass (Singh, 2013). Today ethanol is used to run automobiles blended with gasoline (Somma *et al.,* 2010). The main countries producing biofuels are Canada, USA, Brazil, Argentina, Colombia, France, Germany, China, India Indonesia, Malaysia, Thailand and Australia (Sorda *et al.,* 2010).

1.2 Challenges of cellulosic ethanol production

The current production cost of cellulosic ethanol is high due to the cost of pretreatment and the costs of enzymes produced from microbial fermentation. For example, it has been estimated that cellulases produced from microbial fermentation contribute up to 4-8 cents per liter to the cost of ethanol production and the cost of biomass feedstocks raw material including the pretreatment cost contribute between 13-21 cents per liter of ethanol (Chapple *et al.,* 2007). One of the main challenges of cellulosic ethanol production is the inefficient technologies for the conversion of biomass into fuel ethanol that often have high energy consumption during pretreatment and this eventually results in overall negative energy balance. For example, it was estimated that direct burning of switchgrasses (10 t/ha) yields a 14.6 fold increase in energy outputs over inputs, while conversion of switchgrassess to

ethanol required 45% more energy input over outputs resulting in net negative energy (Pimentel and Patzek, 2005). Pretreatment using low cost dilute sulphuric acid requires addition of substantial amounts of sodium hydroxide to neutralise the hydrolysate and involves proper deposition of produced salts which costs energy. Furthermore, ethanol is recoved through distillation a process that involves energy cost. Hence the future of cellulosic ethanol production depends upon the use of efficient pretreatment technology coupled with reduction of enzyme cost. Overcoming both of these challenges would reduce the cost of cellulosic ethanol production.

1.3 Types of Biofuels

1.3.1 First generation biofuels

First generation biofuels are made from agricultural commodities such as: sugar, starch and vegetable oils. Biodiesel can be produced from oil crops such as; canola, *Jatropha, Pongamia, Moringa, Hura crepitans* and animal fats such as tallows. Bioethanol can be produced by the fermentation of sugars from the stems of sugar-cane (*Saccharum officinarum*) and sugar-beet roots (*Beta vulgaris*). Alternatively starch present in grains (e.g corn, barley, wheat, oats, and sorghum) can be hydrolysed into glucose which is then fermented into ethanol. The European Union has the largest first-generation biodiesel facilities and the United States of America and Brazil are the two main countries that produce first-generation bioethanol (Odling-Smee, 2007). The production of ethanol from sugarcane in Brazil is more cost competitive than the ethanol produced from corn in the USA (Xavier, 2007).

The direct consequence of using first generation biofuel produced from corn grains /starch and sugar from sugarcane as feedstock is that it impacts on food and animal feed industries, thereby increasing the food prices (Pimentel and Patzek, 2005). Furthermore, growing these crops with high carbohydrate content requires continuous input of fertilizers and insecticides that have negative impacts on ecosystems and biodiversity. The cost involved in the operation of farming machinery, pumping water for irrigation and drying of corn kernels all contribute to the final production cost. Life-cycle analysis indicates that the corn based ethanol production has net CO_2 emission rather than being carbon neutral, which suggests that first generation biofuels are not suitable options as an alternative to petroleum based fuel (Hill *et al.,* 2006). Therefore, first generation biofuel is neither scalable nor sustainable (Cleveland *et al.,* 2006), which drives the search for alternative fuels that support the running of existing fuel engines.

1.3.2 Second generation biofuels: Cellulosic ethanol

Second generation biofuels, chemically identical to first generation fuels, but are called advanced biofuels as they are derived from hydrolysis of several types of biomass feedstocks into simple sugars which then fermented into ethanol. Cellulosic ethanol does not compete with food as it is produced from inedible parts of plants that contain cellulose, hemicellulose and lignin. Global primary production of biomass is estimated to capture 4,500 EJ of solar energy each year and generation of 540 EJ of energy from biomass would be enough to meet almost all of the world's total transportation energy demand (Ladanai and Vinterbäck, 2009). Biomass for ethanol production can be obtained from waste materials from agriculture and forestry practices or from dedicated biomass plants such as *Miscanthus*,

switchgrass, poplar, willow, pinewood and also from municipal solid waste (Figure 1.1). For example, plants like *Miscanthus* can grow in arid soil and do not need expensive agricultural inputs and can be grown over a wide range of environmental conditions (Mishra *et al.,* 2013). Hence, cellulosic ethanol protentially could support global transportation energy needs if the challenges associated with biomass conversion are solved appropriately.

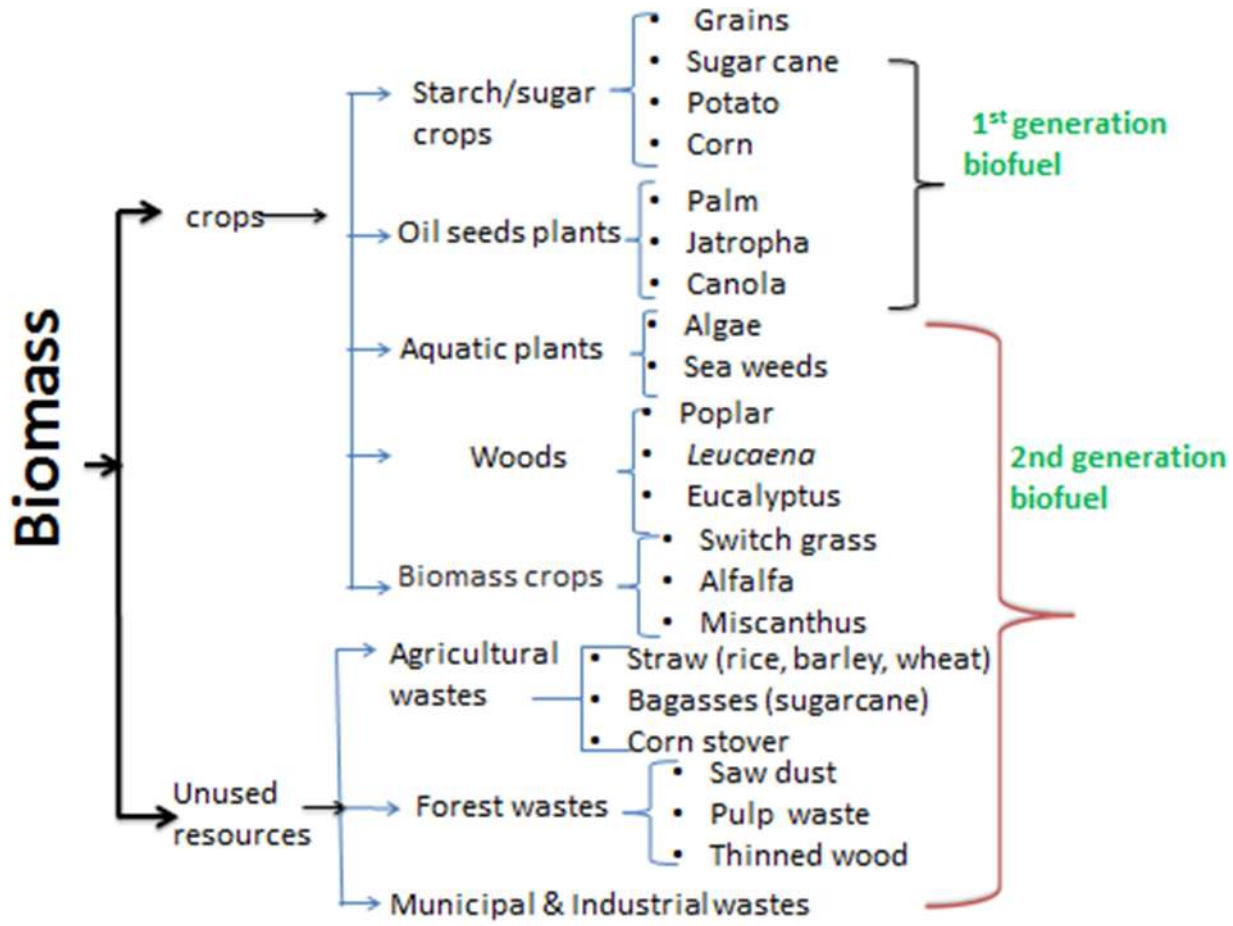


Figure 1.1 Biomass sources feedstock used for biofuel produc*tion*

1.3.3 Biomass recalcitrance: Principal challenge of second generation biofuel production

The term “biomass recalcitrance” is defined as the natural resistance of plant cell walls to microbial and enzymatic deconstruction (Himmel *et al.,* 2007). The complex organisation of the cell wall presents a formidable challenge in breaking down the

recalcitrant cellulose microfibrils that are embedded within the hemicellulose, lignin, and pectin (Figure 1.2). The walls of growing plant cells are called primary cell walls. The new layer deposited between primary cell wall and the plasma membrane is called the secondary cell wall and is made of cellulose, lignin and glycoprotein (Oda and Fukuda, 2012). The secondary cell wall consists of a middle lamella, and three secondary walls (S1, S2 and S3). The three layers of the secondary wall are built up by lamella formed by ordered, parallel cellulose microfibrils embedded in lignin and hemicellulose (Figure 1.2).

Current production methods for cellulosic ethanol are very expensive due to the technical hurdles associated with the biomass recalcitrance (Figure 1.2). The scope of production of carbon neutral and cheaper cellulosic ethanol depends upon the development of new biofuel technologies that convert the biomass of fast growing plants and perennial grasses (Figure 1.1) into simple sugars. The projected long term goal is to generate cheaper and carbon neutral cellulosic ethanol from abundant lignocellulosic feedstocks with improved pretreatment processes and by developing inexpensive enzyme production systems (Dunn *et al.,* 2013).

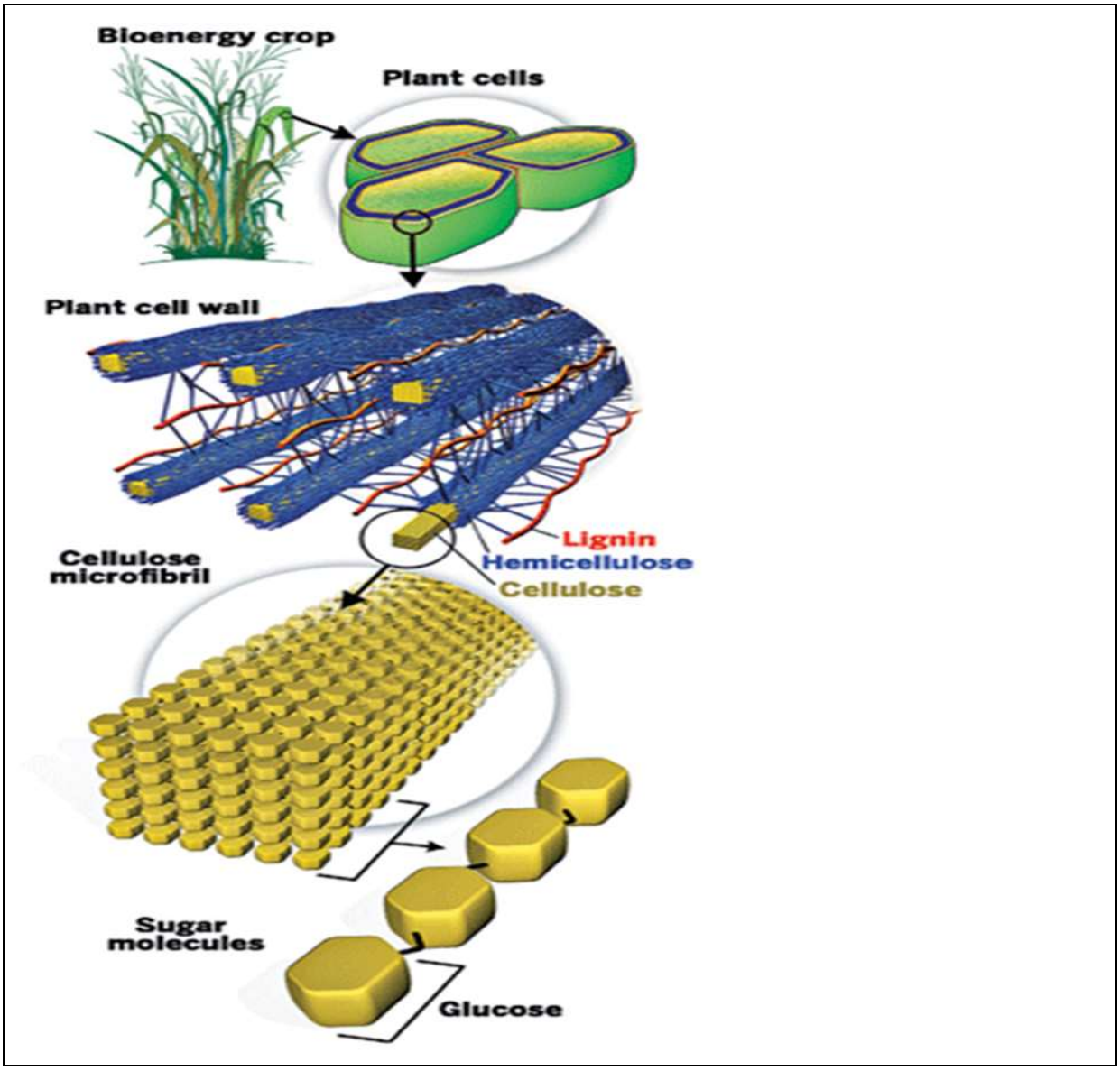


Figure 1.2 Plant cell wall structure showing cell wall components: lignin, hemicellulose and cellulose. Cellulose mirofibrils separated after the pretreatment process become accessible to enzymatic hydrolysis and subsequently release simple sugar molecules (Wyman and Yang, 2009).

1.4.Cellulose

Cellulose is the most abundant biopolymer on earth with estimate of an annual production up to $1.5x10^{12}$ t (Klemm *et al.,* 2005). The cellulose portion of the plant

cell wall varies between 35 to 50% of the total dry biomass and contains a linear chain of β-D-glucopyranose residues linked by β-1,4 glycosidic bonds. Cellulose produced by plants consists of about 50-90% of the polysaccharide chains aligned in parallel arrays, with the hydrogen bonds between the chains forming the microfibrillar crystalline structure (Wyman, 1996) and the remaining portion is less ordered and is called amorphous cellulose (Figure 1.2). The microfibrillar structure of crystalline cellulose is recalcitrant to enzymatic hydrolysis (Jacobsen and Wyman, 2000). The rate of hydrolysis by fungal cellulases are typically 3-10 times faster than ruminal bacteria cellulases when amorphous cellulose was used as substrate instead of highly crystalline cellulose substrate (Lynd *et al.,* 2002). Hence there has been substantial demand of fungal cellulases for cellulosic ethanol production.

1.4.1 Hemicellulose

Hemicelluloses are about 20-50% of the plant cell wall which are linked with β-1,4 backbones of mannans, xyloglucans, xylan and glucomannans. The hemicelluloses are linked with cellulose by C-C and C–O bonds and thus require a certain degree of thermal energy for dissociation. The main role of hemicellulose is to tie with cellulose microfibrils that limit the cellulases contact on substrate cellulose during hydrolysis (Figure 1.2).

1.4.2 Pectin

Pectins are mostly made up of rhamnogalacturonan I and rhamnogalacturonan II, arabinan, galactan, and arabinoglactan. In dicot plants pectin is approximately 35% of the total dry weight and in monocot plants they are much less abundant. Their

function is to help bind cells together, provide cell wall porosity, adjust pH and maintain ion concentration of cells (Caffall and Mohnen, 2009). Pectin oligosaccharides induce lignification (Robertsen 1986) and accumulation of protease inhibitors (Bishop *et al.,* 1984) in plant tissues. Pectin can be degraded with the formation of reducing groups by acid hydrolysis (at pH 2.5-4.5). Some fungi (such as *Aspergillus niger*, *A. oryzae*, *Penicillium chrysogenum*, *Fusarium graminearum*/*Gibberella zeae)* produce pectin degrading enzymes (pectin lyase, pectate lyase, pectin methylesterase ezymes, pectin depolymerase and rhamnogalacturonan acetylesterase) (van den Brink and de Vries 2011).

1.4.3 Lignin

Lignin is present in between the cellulose, hemicellulose and pectin components in cell walls. Lignin is almost non-existent in the primary cell wall, but the secondary cell wall contains lignin which comprises about 10—25% of the total dry weight of the plant. Lignin is a complex polymer made of monolignols or phenylpropanoids. Lignin links with the various carbohydrates molecules (such as cellulose and xylose) present in the plant cell wall via ester-ester bonds and phenyl-phenyl bonds. The presence of lignin adds strength and rigidity to the cell wall. Lignin protects the plant cell against the pathogen invasion; it gets deposited at pathogen wound sites. Hence lignin is a major barrier for enzymatic hydrolysis of the cellulose in the production of cellulosic ethanol. Cellulose and hemicellulose are important sources of fermentable sugars, but most microbes cannot ferment lignin. However, lignin could be fermented by utilization of certain lignin degrading fungi such as *Phanerochaete chrysosporin*

and *Trametes varcicolor* (Staszczak, 2008) produce lignases, manganese dependent peroxidases and lignin peroxidases (Weng *et al.,* 2008, Higuchi, 2006)**.**

1.5 Overview of cellulosic ethanol production

The important steps necessary in the production of the cellulosic ethanol are shown in Figure 1.3 (Sticklen, 2008). The biomass are depolymerised through chemical and/or physiochemical pretreatment, production of enzymes through microbial fermentation, successive addition of enzymes for hydrolysis of pretreated biomass to fermentable sugars and the subsequent conversion of these sugars into ethanol (Hasunuma and Kondo, 2012). The proposed consolidated bioprocessing (CBP) (a single bioreactor that contains microbes capable of hydrolysis and fermentation steps) was designed to reduce the cost and commercially-viable way to produce cellulosic biofuels (Lynd et al., 2005). However, no economically feasible CBP organism has so far been developed yet.

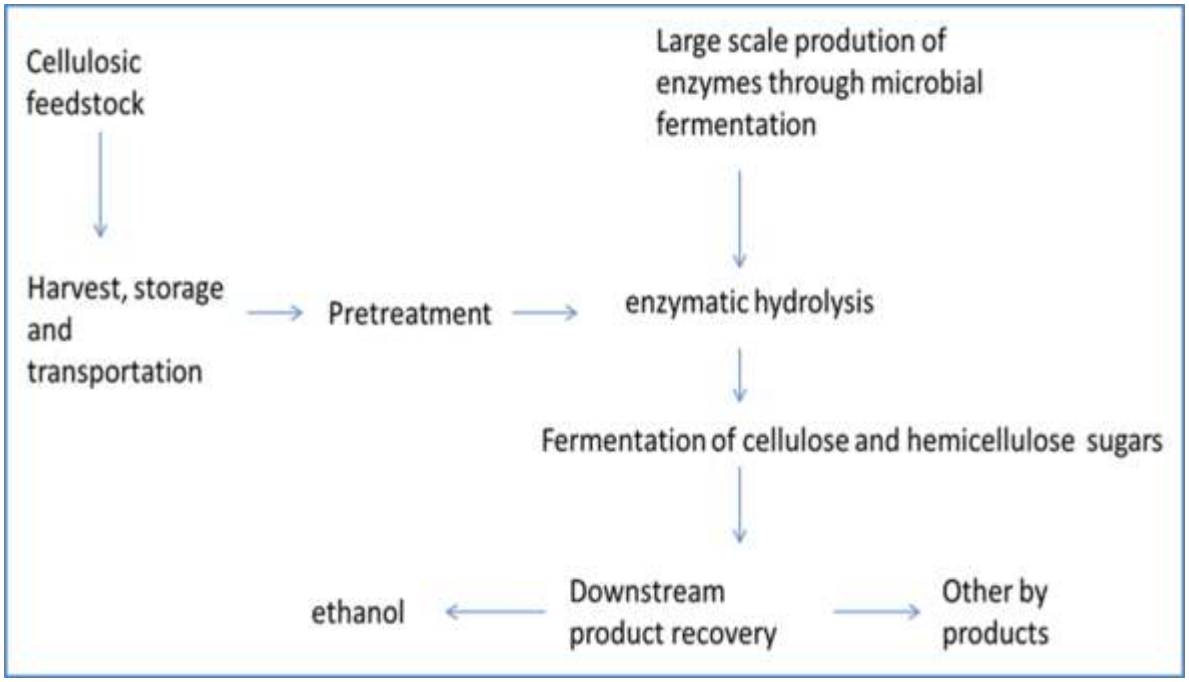


Figure 1.3 Overview of cellulosic ethanol production. Revised form of flow chart showing the steps in the production of cellulosic ethanol from feedstock crops (Sticklen, 2008).

1.5.1 Biomass pretreatment technology

Mechanical grinding helps in size reduction to 20-40 μm in diameter (Hoeger *et al.,* 2013), increases surface area or pore size of the materials and makes the cellulose accessible for saccharification (Hoeger *et al.,* 2013). Pretreatment helps to alter the inherent structure of the lignocellulose and separates cellulose from the matrix of ligin and hemicellulose, reduces cellulose crystallinity, and increases the availability of the specific surface area of cellulose substrate for enzymes to act upon cellulose and hemicellulose. The pre-treatment process accounts for about 20% of the ethanol production cost (Yang and Wyman, 2008, Mosier *et al.,* 2005). Common pretreatment methods include application of heat, enzymes or acids that remove the polymers from the cellulose core prior to hydrolysis. To date several types of pretreatment process are available such as; dilute acid hydrolysis, mechanical

pretreatment, steam explosion (Mabee *et al.,* 2006), supercritical carbon dioxide pretreatment (Kim and Hong, 2001), ammonia fibre explosion (Lau and Dale, 2009), biological pretreatment (Balat *et al.,* 2008) and ionic liquids (Da Costa Lopes *et al.,* 2013). Ammonium fibre explosion (AFEX) has been the most efficient preatment process with fewer chemical by-products that inhibits fermentation. This process also leaves sufficient nutrients in the pretreated stover that help support vigorous growth of the industrial strain of *S. cerevisiae* and can achieve an ethanol yield concentration of 40 g/l (Lau and Dale, 2009).

Autohydrolyis through steam explosion or thermochemical treatment of pre-chopped woody lignocellulosic biomass helps to separate individual cellulose fibres from the other cell wall matrix (Mabee *et al.,* 2006), increases hydrolysis of hemicellulose and promotes delignification processes (Jeoh, 1998). Pretreatment with acid hydrolysis followed by enzymatic hydrolysis of lignocellulosic biomass improves the release of fermentable sugars as compared with the use of other pretreatment methods (Kumar *et al.,* 2009).

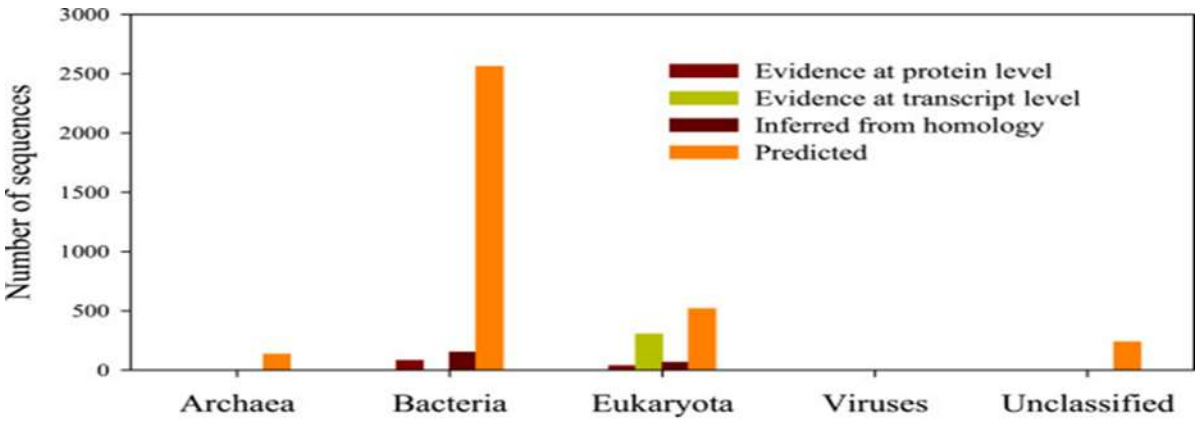
1.5.2 Economic analysis of the cellulosic ethanol production utilising the cellulases produced via microbial fermentation

It has been estimated that the production of cost effective cellulases from *T. reesei* need to be improved by four fold to make this approach to the production of fuel ethanol economically competitive (Lynd *et al.,* 2005). The energy budget of cellulosic ethanol production should include the cost of the biomass raw materials (Balat *et al.,* 2008, Dwivedi *et al.,* 2009), service cost (inputs, labour, farming machinery, transportation and pre-treatment) (Pimentel and Patzek, 2005), pretreatment and

fermentation cost (Sissine, 2007, MCDonaldK.A, 2012). Recently the cost of enzymes, pre-treatment and fermentation have fallen significantly, but the costs of cellulosic ethanol still have to be reduced if they are to be competitive in the market (Isola, 2013). Hence production of low-priced cellulases reduces the cost of cellulosic ethanol production.

1.6 Cellulase producing organisms

Once pretreatment is completed the cellulose thus separated from the lignin, pectin and hemicellulose matrix is then available for enzymatic hydrolysis. In nature a variety of organisms produce sets of enzymes to convert lignocelluosic biomass into simple sugars (glucose) and other by-products. Cellulases are produced by numerous microorganisms including bacteria, fungi and protists, and by other multicellular organism such as termites (insects), abalone (a mollusc) and a sea squirts (tunicates) (Reese, 1956). Cellulases from



Actinobacteria	546
Bacteroidetes	169
Cyanobacteria	17
Fibrobacteres	23
Firmicutes	714
Proteobacteria	1006
Spirochaetes	88
Thermotogae	14
Verrucomicrobia	18
Others	40
Environmental samples	163

Alveolata	18
Amoebozoa	23
Fungi	425
Haptophyceae	1
Ichthyosporea	1
Metazoa	180
Parabasalia	26
Rhodophyta	3
Stramenopiles	9
Viridiplantae	241

Figure 1.4 Summary data of cellulase producing organisms as listed in UniPort Knoweledge database (Yan and Wu, 2013).

Archaea, bacteria, fungi and viruses along with the unclassified sources are listed in the Figure 1.4 (Yan and Wu, 2013). There are about 4,101 cellulases listed in UniProtKB, 133 from Archaea, 2799 from bacteria, 928 from Eukaryota, 2 from viruses and the remaining 239 unclassified (upper panel of Figure 1.4) (Yan and Wu, 2013). Also the CAZY database (http://www.cazy.org) contains sequences based on families of GHs which includes about 14,000 putative bacterialenzyme sequences from families GH5, 6, 8, 10, 11, 26, 39, 43, 44, 48, 51, and 53 (Wei *et al.,* 2009). Usually the cellulases produced from aerobic bacteria and fungi are free enzymes that work synergistically and cellulases from certain anaerobic bacteria form complex structures called cellulosomes (Lynd *et al.,* 2002). Though there are a variety of

organisms that can degrade plant biomass, commercialisation of cellulases production capabilities of these organisms had not been determined fully which open many research questions and possibilities for further investigations.

1.6.1 Cellulases system

The cellulolytic systems are divided into two types which are the free enzymes and multienzyme complexes. Both cellulolytic systems are described further in brief.

1.6.1.1 Free enzymes

The free enzymes are produced by filamentous fungi and are of three main enzyme types; endoglucanases or endo-1-4-β-glucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Figure 1.5A). Endoglucanases attack the cellulose fibre randomly; at multiple internal sites in the amorphous regions of the cellulose fibre and also opens-up sites for subsequent attack by the cellobiohydrolases. Cellobiohydrolases cleave cellobiose from the ends of the glucan chain. Thus, cellobiohydrolases can hydrolyze highly crystalline cellulose. The β-glucosidase hydrolyzes cellobiose and sometimes hydrolyzes short chain oligosaccharides to glucose.

1.6.1.2 Bacterial multi-enzyme complexes: cellulosome

Cellulosomes are large extracellular enzyme complexes produced by certain anaerobic bacteria such as *Clostridium, Acetivibrio, Bacteroides* and *Ruminococcus*

and anaerobic fungi such as *Anaeromyces mucronatus, Orpinomyces* sp. These organisms can degrade cellulose, hemicellulose and pectin of the plant cell wall via the production of a cellulosome complex (Doi *et al.,* 2003, Doi, 2008). Typical cellulosome subunits called cohesins that have enzymatic binding sites and non-enzymatic scaffolding proteins that fit catalytic subunits into the multiple enzyme complexes. As illustrated in Figure 1.5B, the dockerin domains present in the enzyme subunit interacts with the cohesin domains of the scaffoldin subunits. The cellulose binding domains (CBM) present in the scaffoldin help anchor the cellulosome to its substrate.

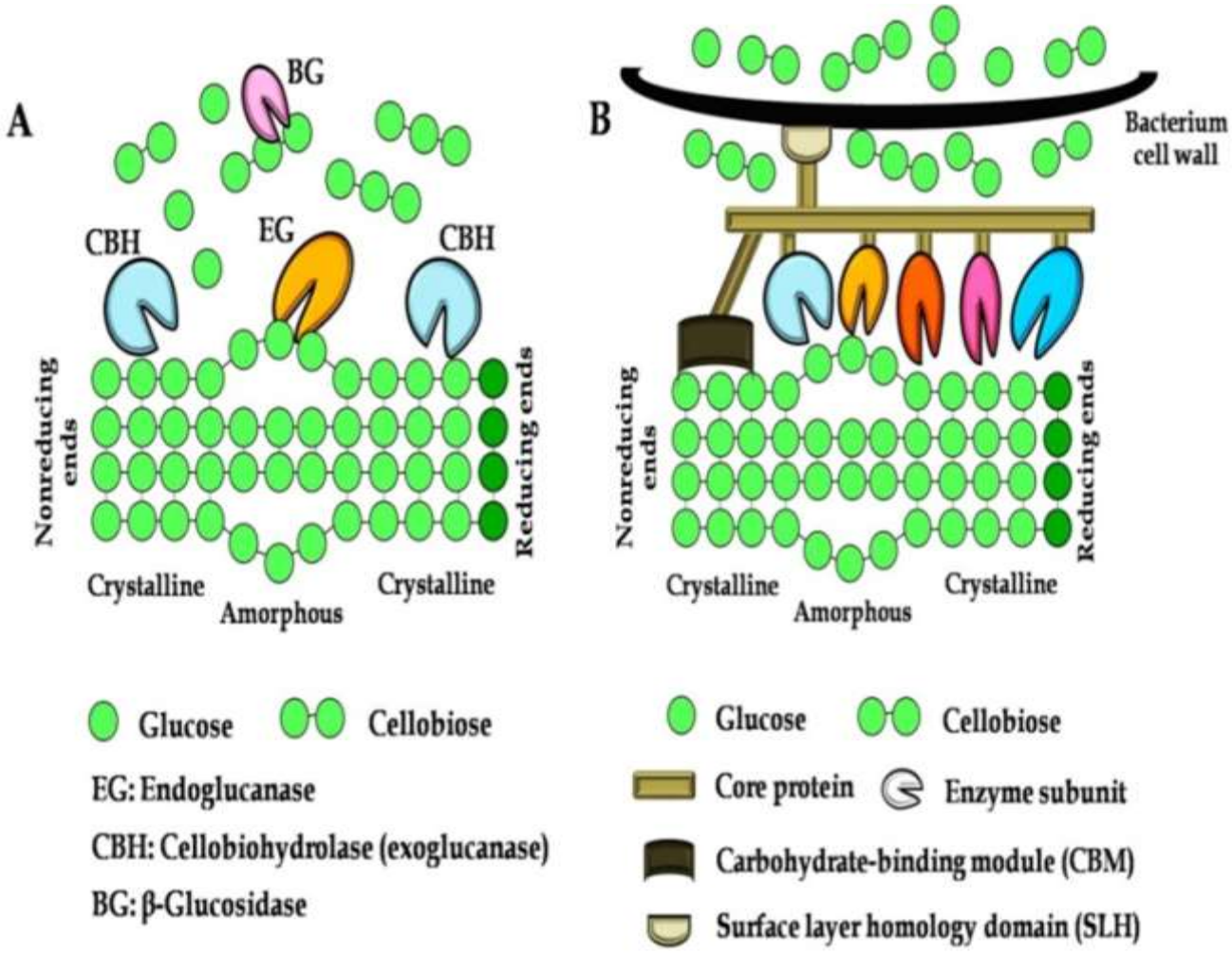


Figure 1.5 Modified verson of schematic representation of the hydrolysis of amorphous and microcrystalline celluloses by (A) non-complexed and complexed (B) cellulase systems (Lynd *et al.,* 2002, Ratanakhanokchai *et al.,* 2013). The reducing and non-reducing ends are shown as above.

1.6.2 Industrially important cellulolytic fungi

Although cellulolytic capabilities are found in Ascomycetes, Basidiomycetes, Deuteromycetes and Zygomycetes, two filamentous fungal groups Sordariomycetes (*Trichoderma*) and Eurotiomycetes (*Aspergillus*) are commonly used for the commercial production of cellulases and hemicellulases (Lambertz *et al.,* 2014, Duan and Feng, 2010). The production of cellulases is an energy-consuming process and

these fungi only produce it when needed to utilise plant polymers as an energy and carbon source (Amore *et al.,* 2013).

1.6.2.1 Trichoderma *reesei cellulases*

The cellulolytic property of *T. reesei* was discovered after examination of the cause of rapid deterioration of cotton fabric of US Army servicemen uniforms from Bougainville Island during World War II and strain QM6a was named after the principal investigator Elwyn T. Reese (Reese, 1976).

Table 1.1 lists the three types of cellulolytic enzyme produced by *T. reesei;* which includes two cellobiohydrolases (CEl7A or CBHI and CEl6A or CBHII), eight endogluconases (CEl5A, CEl5B, CEl7B, CEl12A, CEl61A, CEl45A, CEl61B and CEl74A) (Nutt, 2006) and seven β-glucosidases (CEl1A, CEL1B, CEL3A, CEL3C, CEL3D, CEL4E) (Seiboth *et al.,* 2011). There are also four endoxylanases produced by *T. reesei* (Jørgensen *et al.,* 2007). The three main enzymes that act synergistically to catalyse the complete hydrolysis of cellulose into glucose are; endogluconase (β-1, 4 glucanohydrolase, E.C.3.2.1.4), cellobiohydrolase (CBH, β-

1,4-D-glucancellobiohydrolase, E.C.3.2.1.91) or exoglucanase and β-glucosidase (E.C.3.2.1.21) (Grassick *et al.,* 2004). These cellulase families share the common protein fold, have common catalytic sites and the same reaction mechanism (Seiboth *et al.,* 2011). However, enzymatic activity varies among family types. Both CBHs and endogluconases (EGs) are found within the same family 7 CEL7A and CE

Table 1.1 The cellulase system of T. reesei (Seiboth et al., 2011, Nutt, 2006). Note:* GPI glycosylphosphatidylinositol, # no hydrolytic activity (Harris *et al.,* 2010*)*

GH Family	CAZY nomenclature	Previous designation	Cellulase type	Mol. Wt	Amino acids	Position of CBM	Isoelectric point
1	CEL1A	BGL2	β-glucosidase	114	466	-	-
1	CEL1B	-	β-glucosidase	-	484	-	-
3	CEL3A	BGL1	β-glucosidase	71	744	-	-
3	CEL3B	-	β-glucosidase	-	874	-	-
3	CEL3C	-	β-glucosidase	-	833	-	-
3	CEL3D	-	β-glucosidase	-	700	-	-
3	CEL3E	-	β-glucosidase	-	765	-	-
5	CEL5A	EG2	Endoglucanase	50	397	N	5.5
5	CEL5B	-	Endoglucanase		438	GPI anchor*	-
6	CEL6A	CBHII	cellobiohydrolase	53	447	N	5.9
7	CEL7A	CBHI	cellobiohydrolase	57	497	C	3.9
7	CEL7B	EG1	endoglucanase	55	436	C	4.5
12	CEL12A	EG3	endoglucanase	25	218	-	7.5
45	CEL45A	EG5	endoglucanase	36	270	C	2.9
61	CEL61A	EG4	endoglucanase	55	344	C	-
61	CEL61B	-	Endoglucanase #	-	249	-	-
74	CEL74A	EG6	Endoglucanase/ xyloglucanase	-	818	C	-

such as CBHI,CBHII and E1 produced by *T. reesei* (Zhang and Lynd, 2006). The low turnover rate of CBH with reduced specific activity during cellulose hydrolysis is due to the inhibition by the cellobiose and the addition of β-glucosidase (BG) has been found to improve inhibition and increase the activity of cellobiohydrolases (Jalak and Väljamäe, 2010). Atomic force microscopy (AFM) analysis of the structural change of the cellulose acted upon by addition of CBHI suggests that exocellulases selectively hydrolyze the hydrophobic faces of cellulose (Liu *et al.,* 2011). Another factor that impacts on cellulase performance in *T. reesei* is posttranslational modification (e.g. phosphorylation, proteolytic processing, disulphide bridge formation or attachment of GPI-anchor and O –linked glycoproteins) (Jeoh *et al.,* 2008).

1.6.2.2 Termite gut protozoan cellulases can digest wood

Termites are soft-bodied social insects that can chew wood to digest lignocellulose and convert it into sugar, hydrogen, acetate and nitrogen (Allardyce and Linton, 2012, Warnecke *et al.,* 2007). Termites are classified into two types: the lower termites and the higher termites (Warnecke *et al.,* 2007); higher termite's exhibit foraging feeding behaviour, feed on the various materials and build nesting sites. Higher termites produce cellulases and gut protists are usually not part of the digestion processes. Lower termites can feed on wood with the lignocellulose materials partially digested by endogenous endoglucanase and further digestion occurs in the hindgut through cellulases produced by gut protists. Termite cellulases contain a single domain catalytic site (Li *et al.,* 2011).

1.7 Cellulase structure and function

The structural and functional domains of cellulases are folded autonomously to establish the discrete unit identity (Henrissat *et al.,* 1998). There are three modules: the catalytic domain (CD), the carbohydrate-binding module (CBM) and the linker (Figure 1.6). Consistent with

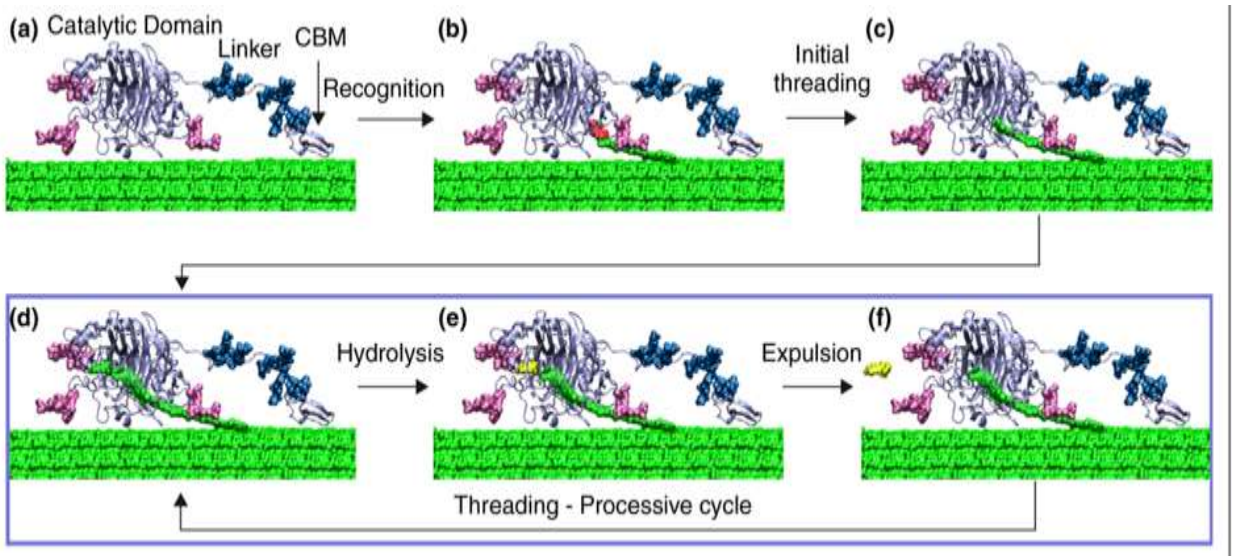


Figure 1.6 Schematic representation of binding mechanism of cellobiohydrolase from *T. reesei* (Quiroz-Castañeda and Folch-Mallol, 2013). a. Structure of CBHI and cellulose (in green), b. recognition of cellulose by the cellulose binding domain (CBM), followed by c. initial threading which subsequently leads into d. hydrolysis, e. and f. the product expulsion steps. The cellulose chain top layer is in green, the cellulose chain bound to the active site in catalytic domain is represented in red and blue represents alpha helix and gray β-sheets of the CBHI.

this *Trichoderma* cellulases have a CD and a CBM linked by a highly glycosylated linker peptide (Receveur et al., 2002). The CBM has a conserved aromatic residue that mainly interacts with glucopyranose rings of the substrate.

1.7.1 Catalytic module

A catalytic module is defined as the structural portion or region of amino acid sequence of multidomain enzymes that possesses the catalytic activity (Miles, 2010). The three-dimensional structure of CBHI from *T. reesei* was resolved to 1.8 Å resolution and it was found that the catalytic domain contains a 40 Å long active site tunnel (Divne *et al.,* 1994). There are 434 amino acids in CBHI of *T. reesei*, about one third consists of 2 antiparallel β-sheets stacked with β-sandwich and the remaining loops are connected with the β-strands. There are about 10 disulphide bridges in the CBHI structure and 9 of them stabilise the loops. The 2 β-strands have 7 concave and 8 convex faces that are antiparallel to each other (Divne *et al.,* 1994). The tunnel consists of binding sites for the cellobioside derivatives with a $(\alpha/\alpha)_6$ barrel-like structure, with a six inner and six outer α helix structures called GH 8, 9 and 48. The (β/α) barrels consist of eight repeating units of β/α module surrounded by eight α helices, such as GH 5 and 10. The β jelly rolls expands with three antiparallel β strands connected by hairpins followed by longer connection by the fourth strand which lies adjacent to the first. Two β sandwiches are packed together face-to-face that twist and coil to form a close structure 7 glycosyl binding sites from A to G. The ligand O-idobenzy-1-thio-β-D –cellobioside (TBTC) binds to the entrance C group in A and glycosyl unit in B (Divne *et al.,* 1994). Families of cellulases are classified based on the amino acid and folding similarities of the catalytic modules (Henrissat, 1991). Table 1.2 shows the series of folding of catalytic cellulases of both bacteria and fungi, which is mostly of endoglucanase families (Wertz *et al.,* 2010). Note that GH-7 in Table 1.2 represents only fungal cellulases and GH-8 represents only bacterial cellulases.

1.7.2 Carbohydrate-binding module (CBM)

A CBM is defined as “contiguous amino acid sequences with discrete folds within the modular structures of carbohydrate active enzymes and cellulosomal scaffoldings” (Boraston *et al.,* 2004). The CBM was previously termed the cellulose binding domain (CBD) (Lehtiö, 2001). The CBM size varies from 4-20 kDa, usually present at one end of the protein sequence and binds to carbohydrate chain for its enzyme immobilisation into the substrate (Hilden and Johansson, 2004). The binding of the CBM with crystalline cellulose usually occurs through aromatic polar residues such as tyrosine and tryptophan at a distance of 10.3 Å from the flat cellulose chain (Tomme *et al.,* 1995).

1.7.3 Linker region

A linker is the region of amino acids of multidomain cellulases connecting catalytic domain and the CBM (Figure 1.6.a). The roles of the linker are not well understood. The linkers that connect a discrete catalytic domain and cellulose binding domain of microbial cellulases and xylanases are rich in proline and hydroxyamino acids. It has been predicted that glycans on the linkers potentially impart rigidity to the cellulases, help in preventing proteolysis of the enzymes and entrance binding to the cellulose surfaces (Payne *et al.,* 2013). Deletion of the Pro-Thr box in the linker alters the confirmation and orientation of both catalytic and the CBM (Shen *et al.,* 1991).

Table 1.2: Folding of catalytic module of different cellulases from bacterial and fungal origin.

Glycosyl Hydrolase (GH) family (clan)	Fold	Enzyme type	Organism Source
5 (A)	$(\beta/\alpha)_8$ barrel	Mainly endoglucanases	Bacteria, fungi
6	β/α	Endoglucanases and CBHs	Bacteria, fungi
7 (B)	β jelly-roll	Endoglucanases and CBHs	Fungi
8 (M)	$(\alpha/\alpha)_6$ barrel	Mainly endoglucanases	Bacteria
9	$(\alpha/\alpha)_6$ barrel	Mainly endoglucanases	Bacteria, fungi
12 (C)	β jelly-roll	Endoglucanase	Bacteria, fungi
45	β barrel	Endoglucanases	Bacteria, fungi
48	$(\alpha/\alpha)_6$ barrel	Processive, endoglucases and CBHs	Bacteria

1.8. Enzymatic hydrolysis of cellulose

The synergistic actions of CBHI, E1 and CBHII on amorphous cellulose are illustrated in Figure 1.5A. The CBHI acts from the reducing end, CBHII from nonreducing end, E2 from nonreducing end and E1 acts randomly (Quiroz-Castañeda and Folch-Mallol, 2013). CBHI contains tunnel-like active sites that accept cellulose (the substrate) chain through their terminal units. Both CBHI and CBHII act by threading cellulose chain ends, releasing cellobiose in a sequential manner (Grassick *et al.,* 2004). Endoglucanase synergise with CBHI to hydolyse

cellulose from the middle of the chain into small oligosaccharides. Finally, the cellobiose is hydrolysed by β-glucosidase (E.C.3.2.1.21) into glucose.

1.8.1 Binding mechanism of cellulases to cellulose

The steps in binding mechanism of cellobiohydrolysis are: CBHI cellulose recognition, initial threading, hydrolysis, and product expulsion for the free CBHI (Figure 1.6). The CBHI recognises the cellulose substrate and initiates the threading of the cellulose chain. CBHI binds with substrate cellulose (crystalline structure) through its CBM then from the reducing end of the cellulose cleaves the glycosidic bonds, which undergoes a conformational change before moving along the cellulose chain. The last step is product expulsion and threading of another cellobiose.

1.9 Heterologous expression of cellulases

Large amounts of cellulases are required for the conversion of biomass to ethanol. For example it has been estimated around 15 g of cellulases are required for the hydrolysis of one kg of biomass (Montague *et al.,* 2002). One of the major challenges in cellulosic ethanol production is to overcome the issue of production costs of cellulases and their enzymatic performance. Development of low-cost enzymes remains a priority for the cellulosic production industry and one way to overcome this challenge is through optimisation of recombinant expression systems. Various scientific approaches for cellulases improvement have been proposed such as; recombinant enzyme production by rational design, enzyme improvement by directed evolution, consolidated bioprocessing, strain improvement of fungal host for

hyper-production of cellulases and use of alternative heterologous hosts for cellulase production (Jeoh *et al.,* 2008).

Even though a genetically engineered *T. reesei* strain has been reported to produce 20-100 g of crude cellulases per litre of culture medium (Wilson, 2009, Peterson and Nevalainen, 2012), continuous production of cellulases using this approach is limited because of the special culturing and induction condition requirements. Thus, production of these cellulases in native host strain or in heterologous production system are not economical due to product inhibition, costs involved in purification and downstream processing. Additionally, cellulose hydrolysis involves a set of different enzymes that need to act in concert at different ratios depending upon the biomass composition (Blouzard *et al.,* 2010, Chundawat *et al.,* 2011). Thus, heterologous expression of cellulases via microbe or plant based production systems are expected to improve the yield and make the immediate goal of production of cellulosic ethanol more likely.

1.9.1 Cellulase expression in bacterial systems

The expression of cellulases in bacteria provides many advantages such as rapid production of recombinant protein and the possibility of engineering multienzyme complexes (*Maki et al.,* 2009). Compared to fungal hosts bacteria have higher growth rates, which helps in the rapid production of recombinant protein. However, a bacterial system having these traits that could potentially be used for conversion of cellulosic biomass into component sugars are yet to be characterised. That said gram negative bacteria such as *E. coli, Klebsiella oxytoca* and *Zymomonas mobilis*

can be engineered genetically for heterologous expression of cellulases (Dien *et al.,* 2003, Maki *et al.,* 2009).

Researchers are also interested in designing a model cellulosomal complex in bacteria especially with cellulases from *T. reesei.* The cellulosomal complex model is expected to help improve our understanding of cohesion function, in analysing synergy between various cellulases and improve the efficiency of cellulosomal complexes (Doi *et al.,* 2003, Maki *et al.,* 2009, Fontes and Gilbert, 2010, Moraïs *et al.,* 2010). To date marginal success has been achieved utilising non-cellulolytic bacteria such as *E. coli* and *Z. mobilis* as heterologous cellulase expression platforms (Doi *et al.,* 2003, Garvey *et al.,* 2013) and there is a need to improve the cellulolytic potential of cellulase producing bacteria, for example using *Bacillus subtilis* (Doi et al., 2003).

1.9.1.1 Expression of cellulases in *E. coli*

E. coli is a Gram-negative, rod-shaped bacterium found in the lower intestine of warm-blooded organisms. *E. coli* has been reported to produce cellulase (Zogaj *et al.,* 2001) and also has been used as a high level production platform of heterologous proteins (Hannig and Makrides, 1998). Among the limitations of using *E. coli* for heterologous cellulase expression is the double membrane system that does not allow cellulase secretion (Gentschev *et al.,* 2002) causing toxic effect to the cells followed by cell growth inhibition and inclusion body formation (Garvey *et al.,* 2013, Tanaka *et al.,* 2011, Jung *et al.,* 2012). Certain strategies, including gene fusion and selection of weak promoters for the expression of most toxic cellulases has been employed to overcome the challenges associated with the *E. coli*

expression system (Zhou *et al.,* 1999). It has been reported that the fusion of CBM of one enzyme with the catalytic domain of another cellulases helps to improve cellulolytic ability of *E. coli* (Murashima and Kosugi, 2003). The problem of plasmid instability and improper disulphide bond formation of recombinant proteins in *E. coli* were corrected by the reducing the growth temperature by 8-10^{o}C (Song *et al.,* 2012).

1.9.1.2 *Zymomonas* expression of cellulases

Z. mobilis is a gram negative, facultative anaerobic, ethanol tolerant bacterium that can utilise high amount of sugar, and grow micro-aerobically. E1 and GH12 from *Acidothermus cellulolyticus* have been expressed in *Z. mobolies* and show some activity (Linger *et al.,* 2010). Thus the possible use of *Z. mobilis* as a host and as a expression platform for cellulases and for biofuel production exists, however, additional research is needed for the host to produce cell wall degrading enzymes (Jung *et al.,* 2012).

1.9.2 Yeast expression of cellulases

Yeasts have been explored as a host for over-expression of fungal and bacterial cellulases. The most commonly used yeasts for the recombinant protein production are; *S. cerevisiae, Pichia pastoris,* and *Kluyveromyces marxianus*. *S. cerevisiae*, known to have a high tolerance to ethanol, has been thoroughly investigated as a host organism for heterologous cellulases expression (Romanos *et al.,* 1992, Jeffries and Jin, 2000).

Most of the important cellulase components of *T. reesei* have been expressed in *S. cerevisiae,* with varying degrees of success. The endoglucanase from *T. reesei* has been shown to express detectable activity suggesting the proper folding of this protein (Lynd *et al.,* 2002). The production of functionally active heterologous CBHs in *S. cerevisiae* is possible but the reported titres of secreted CBHs are relatively low (Haan *et al.,* 2007).

Different methods were used to improve the high-level secretion of cellobiohydrolases from transformed yeast cells to overcome the metabolic burden marked by low levels of mRNA and secreted proteins (Ilmen *at al.,* 2011). However, poor performance may be due to incorrect folding (Boer *et al.,*2000) and hyperglycosylation of CBHI expressed in *S. cerevisiae* (Den *et al.,* 2013). Expression of CBHI in *S. cerevisiae* by fusion of the catalytic domain from *Talaromyces emersonii* and the linker and CBM from *T. reesei* increased yield and activity compared to expression of a native *T. reesei* CBHI (Wei *et al.,* 2014). Researchers are interested to develop cellulase expression systems in yeast, to display single enzymes (Apiwatanapiwat *et al.,* 2011) and multi-enzyme complexes on their cell surface (Ito *et al.,* 2009). Recombinant cellulase expression in yeast has been shown to work successfully utilising the α-mating factor as a secretion signal and α-agglutinin as an anchor protein (Yamada *et al.,* 2013). Other successful expressions of cellulases in yeast includes family 7 glycosyl hydrolases and family 6 glycosyl hydrolase (Den *et al.,* 2007). These studies showed that *S. cerevisiae* can be used effectively for the production of recombinant cellulases as compared with *E. coli* expression system (Young and Robinson, 2014). Future research has been proposed to develop a *S. cerevisiae* strain for surface assembly of a functional mini-cellulsome model and use this to analyse the conversion of biomass into simple

sugars and subsequent fermentation into ethanol (Goyal *et al.,* 2011, Tsai *et al.,* 2010).

1.10 Heterologous expression of termite cellulases

Termites play an important environmental role in the degradation of the lignocellulose component of plants. There are two types of cellulolytic systems in termites, the endogenous cellulases and the cellulases of symbiotic protist origin that coexist within a lower termite gut (Nakashima *et al.,* 2002). The recombinant cellulases derived from termite could be used for degradation of lignocellulosic material during cellulosic ethanol production. Heterologous expression of termite cellulases in a number of organisms has been reported including bacteria, fungi and yeast. The endo β-1,4 glucanase gene from *R. speratus, Nasutitermes takagoensis, Coptotermes formosanus* and *Coptotermes acinaciformis* when expressed in *E. coli* have higher β-glucosidase activity than that produced from native termite species (Ni *et al.,* 2005). When the transcript encoding RsSymEG (an endoglucanase of glycosyl hydrolase family (GHF) 7) isolated from the symbiotic protist of the termite *Reticulitermes speratus*, expressed in *Aspergillus oryzae*, it was found that RsSymEG1 had higher specific activity than previously reported data for GHF7 endoglucanase of *Trichoderma ressei* (Todaka *et al.,* 2010). However, there are no reports of the heterologous expression of termite derived exocellulases in plants

1.11 In planta expression of cellulases

A different approach to overcome cellulase cost is to express in plants rather in other heterologous hosts (Sticklen, 2006, Gray *et al.,* 2009). *In planta* expression of

cellulases had been projected to be more cost effective alternative to generate cellulosic ethanol (Sticklen, 2008, Taylor II *et al.,* 2008, Sainz, 2011). Plant expression system could provide post-translational modification requirements for eukaryotic cellulase expression of large-scale production and plant produced cellulases need not be purified and used directly for cellulases expression (Kusnadi *et al.,* 1997, Twyman *et al.,* 2003). Production of cellulosic ethanol from transgenic plants expressing cellulases could be more economical as it may not require the use of expensive bioreactors and complicated purification steps for cellulases produced via microbial fermentation (Sticklen, 2008, Taylor II *et al.,* 2008, Sainz, 2011, Shen *et al.,* 2012, Jung *et al.,* 2013). Thus, future cellulosic ethanol production may be dependent on transgenic plant biomass containing recombinant cellulases, combined with cellulases produced from fermentation by synthetic cellulolytic microbes. The biomasss of the plants containing recombinant cellulases expressed either in leaves or in kernel/seeds becomes available either after size reduction of biomass step during cellulosic ethanol production or the plant produced cellulases are harvested and added to the biomass of other crops used for the substrate to produce ethanol.

There are several challenges in the heterologous expression of cellulases in different subcellular locations of plants that include glycosylation of extracellular enzymes, and requirement of multiple cellulases for the degradation of the cell wall components. However, it is very important that the production of heterologous enzymes in transgenic plants does not have a deleterious effect on the plant growth (McDonald, 2012, Dai *et al.,* 1999b, Ziegelhoffer *et al.,* 1999). The solution to this problem could either be sequestering the enzymes in a subcellular organelle/space away from the cell wall matrix or use of an inducible expression system to produced

cellulases prior to harvest time so that the produced enzyme doesn't have direct impact on the plant growth and development (Lebel *et al.,* 2008, Taylor II *et al.,* 2008).

Table 1.3 A list of exoglucanases and endoglucanases that have been expressed in plants.

Protein	Source	Host	Site	Yield	Reference
E1 (truncated)	*A. cellulolyticus*	Tobacco	chloroplast	1.36%	(Dai *et al.,* 2000a)
E1	*A. cellulolyticus*	Tobacco	chloroplast	ND	(Jin *et al.,* 2003)
E1 (holoenzymes)	*A. cellulolyticus*	Tobacco	apoplast chloroplast cytosol	0.33% 0.0007 % 0.0006 %	(Ziegelhoffer *et al.,* 2001)
E1 catalytic domain	*A. cellulolyticus*	Tobacco	chloroplast	12%	(Ziegelhoffer *et al.,* 2009)
E1 catalytic domain (truncated)	*A. cellulolyticus*	Tobacco	apoplast chloroplast cytosol	0.4% 0.074% 0.0046 %	(Ziegelhoffer *et al.,* 2001)
E1 (holo-enzymes)-truncated	*A. cellulolyticus*	Tobacco	apoplast	0.25%	(Dai *et al.,* 2005)
E1	*A. cellulolyticus*	Potato	chloroplast	2.6%	(Dai *et al.,* 2000b)
E1 (truncated to catalytic domain)	*A. cellulolyticus*	*Arabidopsis*	apoplast	26%	(Ziegler *et al.,* 2000)
E1	*A. cellulolyticus*	Rice	apoplast	4.9%	(Oraby *et al.,* 2007)
E1 catalytic domain	*A. cellulolyticus*	Corn	apoplast	1.16%	(Ransom *et al.,* 2007)
E1 (truncated to catalytic domain)	*A. cellulolyticus*	Corn	apoplast	2.1%	**(**Biswas *et al.,* 2006)
E1	*A. cellulolyticus*	Corn	apoplast ER vacuole	0.5% 17.9%/6.1% 16/5/6%	(Hood *et al.,* 2007)
E1 (truncated)	*A. cellulolyticus*	Duckweed	cytosol	0.24%	(Sun *et al.,* 2007)
E2 (truncated)	*T. fusca*	Alfa Potato Alfalfa	cytosol cytosol cytosol	0.01% ND 0.1%	(Ziegelhoffer *et al.,* 1999)
E3 (cellobiohydolase)	*T. fusca*	Alfa Potato Alfalfa	cytosol cytosol cytosol	0.002% ND 0.02%	(Ziegelhoffer *et al.,* 1999)
Cel6A	*T. fusca*	Tobacco	chloroplast	2-3%	(Yu *et al.,* 2007)
Cel6B (CBHI)	*T. fusca*	Tobacco	chloropalst	3-4%	(Yu *et al.,* 2007)
EgI (truncated protein)	*Ruminococcus albus*	Tobacco	cytosol	0.1%	(Kawazu *et al.,* 1999)
CelA/Cel6G (shuttled/codon optimised hybrid)	*Neocallimastix patriciarum, Piromyces* spp	Barley	rain endosperm	1.5%	(Xue *et al.,* 2003)
CBHI	*T. reesei*	Corn	apoplast,	17.8/3.2	(Hood *et al.,* 2007)

			ER Vacuole (no activity)	%, 16.3/4% ,	
CBHI	*T. reesei*	Tobacco	leaves and calli	0.11% 0.082%	(Dai *et al.,* 1999b)
CBHI	*T. reesei*	Sugarcane	ER vacuole chloroplast	ND	(Harrison *et al.,* 2011)
TrCel5A	*T. reesei*	Tobacco	leaves	ND	(Klose *et al.,* 2013)
glucuronoyl esterases (GCEs)	white-rot basidiomycete *Phanerochaete carnosa*	*Populus tremula L. x tremuloides Michx.*	Plant cell wall	ND	(Gandla *et al.,* 2014)

Regardless of the challenges with *in planta* expression systems, preliminary research has shown some success in expression of cellulases in plants. For example, apoplast targeted expression of an active *A. cellulolyticus* cellulase, E1 in maize and tobacco has been shown with no detrimental effects on plant growth (Ziegelhoffer *et al.,* 2001, Dai *et al.,* 2005, Biswas *et al.,* 2006). Table 1.3 list active plant produced glycosyl hydrolases that can even release some amount of sugars from the plant biomass (Kawazu *et al.,* 1999, Montalvo-Rodriguez *et al.,* 2000, Ransom *et al.,* 2007, Oraby *et al.,* 2007).

Table 1.3 also compares the accumulation of different cellulases in different cellular compartment of transgenic plants. The highest expression E1 obtained from *Acidothermus cellulolyticus* and CBHI from *T. reesei* accumulate upto 16% TSP in single seed maize (*Zea mays* L.) targeted in the apoplast but still it is not sufficient to fulfill the demand of this enzyme (Hood et al., 2007). Hence there exist the need to develop transgenic plants expressing CBHI at a level sufficient for the hydrolysis of biomass for the production of cellulosic ethanol (Seiboth *et al.,* 2011) and expression of CBHI in the chloroplast might increase the CBHI yield.

1.11.1 Host plants for heterologous expression of cellulases

In an effort to reduce the production cost alternative approaches are being used currently to produce transgenic plants that express cellulases in tobacco (*Nicotiana tabacum*) (Sainz, 2011), *Arabidopsis* (Ziegler et al., 2000), alfafa, *Arabibopsis*, potato, rice and barley (Dai et al., 2000b, Taylor II *et al.,* 2008). Cellulase expression has also been reported in feedstock species, grasses and the fast growing woody biomass species such as *Populus* and *Salix* (Gandla *et al.,* 2014). Tobacco is now routinely used for plastid transformation along with some other related solanaceous species including eggplant (Singh et al., 2010), potato (Sidorov *et al.,* 1999) and tomato (Ruf *et al.,* 2001). The rationale for using tobacco is that it has been possible to use it as a model organism having more detailed information of plastid transformation and it is easy to optimise the desired level of expression of the cellulases.

1.11.2 Expressing cellulases in plants through nuclear transformation

Nuclear integration and expression of cellulases from the host plant remains ineffective to develop higher-level of cellulases products for complete enzymatic hydrolysis of the cellulosic biomass is a requirement (Hood *et al.,* 1997, Streatfield *et al.,* 2002). Nuclear integration of cellulase genes containing the signal peptide for specific subcellular sites such as endoplasmic reticulum, apoplast, vacuole, mitochondria, peroxisomes and chloroplast was found to successfully direct the protein accumulation within specific cellular compartments (Dai *et al.,* 2005, Hyunjong *et al.,* 2006, Hood *et al.,* 2007, Mei *et al.,* 2009, Harrison *et al.,* 2011, Kim

et al., 2010). In *Arabidopisis* E1 endocellulase from *A. cellulolyticus* expression was found to accumulate up to 26% of TSP within the targeted apoplast region following nuclear integration (Ziegler *et al.,* 2000). Expression of CBHI, in corn apoplast was up to 17.8% TSP and 16% TSP in the seeds (Hood *et al.,* 2007) (Table1.4). However, seed based expression of cellulases takes a long time to mature and to harvest as compared to cellulase expression in leaves and stems.

1.11. 3 Expressing cellulases through plastid transformation

Since the first stable plastid transformation in *Chlamydomonas* (Boynton *et al.,* 1988) and in tobacco (Svab and Maliga, 1993) were reported there have been several attempts to explore the plastid transformation of cellulase and hemicellulases genes derived from bacteria and filamentous fungi (Dai *et al.,* 2000a, Dai *et al.,* 2005, Hooker *et al., 2*001, Jin *et al.,* 2003, Hyunjong *et al.,* 2006, Yu *et al.,* 2007, Lebel *et al.,* 2008, Nakahira *et al.,* 2013, Adem *et al.,* 2017). For plastid transformation site-specific homologous recombination between the transformation vector and the plastid genome is required to insert foreign genes in the plastid genomes (Maliga, 2004). After the incorporation of transforming DNA into the plastid genome, repeated selection for a marker is needed before the plant reaches homoplasmy, a state in which non-transformed genomes in the plastid are all replaced with recombinant genomes. The most commonly used selection marker is *aadA,* which encodes aminoglycoside adenyl transferase and that confers spectinomycin resistance (Day and Goldschmidt-Clermont, 2011). Unlike nuclear transformation, transgene integration into the plastome is based on two homologous recombination events taking place between the targeting regions of the transformation vector and the wild-

type ptDNA, ensuring high transformation efficiency of the transplastomic plants (Ruhlman *et al.,* 2010 ; Valkov *et al.,* 2011).

Due to the high copy number of the plastid genome there is the possibility to generate thousands of copies of the transgene per cell via the stable transformation of plastids. For example plastid transformation had been shown to express recombinant protein to levels up to 35-70% of total soluble protein (TSP) (Viitanen *et al.,* 2004, Kumar *et al.,* 2004, Oey *et al.,* 2009). Furthermore, plastid transformation has the advantage of the absence of epigenetic effects such as gene silencing (De Cosa *et al.,* 2001, Dufourmantel *et al.,* 2006), the possibility of multi-gene engineering (De Cosa *et al.,* 2001), and potential high level transgene expression (De Cosa *et al.,* 2001). Plastid based expression may also reduce the level of pleiotropic effects on plant phenotype when toxic transgene products are expressed (Verma and Daniell, 2007).

One of the main advantages of the plastid transformation of cellulases is that ease of transgene stacking to produce multiple enzymes in the same plants and sequestration of the enzyme products within the chloroplasts. The E1 expression from plastid were up to 12% TSP (Ziegelhoffer *et al.,* 2009) and other bacterial cellulases were expressed at a ranges that produced from 2-40% TSP (Gray et al., 2009, Yu *et al.,* 2007, Leelavathi *et al.,* 2003). Since glycosylation appears to be important for protein stability and activity in some fungi (Jeoh *et al.,* 2008), the disadvantages would be lack of glycosylation system in plastids that limits the proper folding of expressed protein. Another disadvantage of these systems is that currently it is not possible to transform the plastid genome of many crops, especially monocots such as rice, wheat, maize and sugarcane. Moreover, studies have shown that

overexpression of cellulases resulted either with slow growth or some detrimental effects in transplastomic plants (Petersen and Bock, 2011, McDonald, 2012).

1.11.4 Challenges of cellulase gene expression in plants

Generation of transgenic plants capable of expression of large amount of recombinant cellulases derived from bacteria, fungi or other protozoan sources will contribute to efficient cellulose deconstruction of the plant biomass (Miles, 2009, Hood *et al.,* 2007, Jørgensen *et al.,* 2007). Heterologous expression of cellulases in plants have several challenges such as; incorrect post-translational modification of the expressed protein (Daniell *et al.,* 2001), premature cell wall degradation and negative impact on the plant growth and development of the transgenic plants (Øbro *et al.,* 2010 & Wilson, 2009) and very high level expression of cellulases in plant may exhaust the chloroplast protein synthesis capacity causing altered plant phenotype (i.e. pigment-deficient and retarded growth) (Castiglia *et al.,* 2016).

1.12 Project overview

Currently all bioethanol production is met by corn starch rather than from cellulosic biomass derived ethanol (Wright and Wimberly, 2013). Ethanol produced from starch or sugar crops does not seem to fit for long term due to the increasing fuel, food, feed, and other needs (Sainz, 2011). The future of cellulosic ethanol is promising as there is abundantly-available inedible cellulose biomass on earth (Ladanai and Vinterbäck, 2009) but the successful production of ethanol at scale with net gain in

energy remains challenging due recalcitrant nature of the biomass (Himmel *et al.,* 2007).

One of the major challenges in making cellulosic ethanol cost-competetive are to reduce production cost of cellobiohydrolases (CBH) which is the dominant enzyme required for hydrolysis of cellulose and its production via microbial fermentation is inefficient thereby increases the costs of cellulosic ethanol production. The plastid expression of CBHI could be useful for increasing the production of recombinant CBHI given the fact that the plastid expressions of other recombinant protein have increase yield (Oraby *et al.,* 2007).

1.13 Research questions

The overall objectives of this project were to:

- to develop transplastomic tobacco plants expressing functionally active exocellulases **(**CBHI from *T. reesei* and excocellulases (Co14), to produce enhanced, lower-cost exocellulase.
- to identify the difference between the phenotype and ultrastructural organization of chloroplasts in the mesophyll of transplastomic tobacco plants expressing these enzymes.

Thus, this project will examine the following questions.

1. Is it possible to generate transplastomic plants expressing functionally active exocellulases?
2. Are there any differences in expression level and activity of CBHI from *T. reesei* and excocellulases (CO14) derived from termite gut protozoan when expressed in plant chloroplast?

1.14 Project Aim

This project was designed to evaluate the feasibility of expressing cellulases in transplastidic tobacco. Genes for two exocellulases, *cbhI* from *T. reesei,* and a novel gene (*co14*) (protozoan cellulase from termite gut) were selected for investigating chloroplast expression. To date there are no reports of either of these enzymes having been expressed in plant plastids.

Chapter 2: Materials and methods

2.1 Growth curve for *E. coli* strains containing *cbhI* in plastid expression vectors

End point quantitation studies are typically used for normalisation and measurement of inhibitory effects on bacteria growth in the LB broth. For determining the concentration of bacteria in the medium absorbance was measured at 600 nm.

Bacterial growth curves were obtained using empty vector in *E. coli* DH5α at three temperatures 37^{o}C, 30^{o}C and 25^{o}C. The negative control was *E. coli* DH5α itself and positive control was *E. coli* containing a *gfp* vector. The starter culture was obtained from a strain storage culture, streaked on Luria broth (LB)-plate (Penicillin 200 mg/L + 0.4% glucose w/v) and grown for 3 d at 25^{o}C. For growth curve analysis of 10 mL cultures with LB broth containing 200 mg/L Penicillin were set up with *E. coli* containing plastid expression cassettes of *cbhI* and *gfp*. The starter cultures for all the samples were at OD_{600} value 0.006. The OD_{600} for all the samples was taken at 4 h intervals up to 24 h (Figure 5.1).

2.2 Plant materials for stable transformation

The plant materials used in this project were *in vitro* shoot cultures of *Nicotiana tabacum cv. Petit Havana* (tobacco) and maintained in MSO (Murashige andSkoog) (MS) medium. Surface sterilised seeds were first germinated in half-strength MS medium and then individual shoots were transferred and maintained in 500 mL MSO medium (Murashige and Skoog, 1962).

2.3 Plant material for transient assays

N. benthamiana seeds were grown in soil less potting mixture (Bunnings, Australia). Seeds of *N. benthamiana* were sown into pot covered with a transparent plastic dome. Seedings were grown at 25^{o}C day/night temperature and 16 h/ 8 h light / dark cycles. After two weeks nitrogen and phosphrous fertilizer at 1.4 g/l were added. At week 4, the plants were transferred into new larger pots to provide adequate space for further growth and plants were maintained until 6-8 weeks for infiltration experiments.

2.4 *Agrobacterium tumefaciens* culture preparation

The *A. tumefaciens* strain Agl1 containing pCAMBIA 1305.2 was subcultured on LB-Agar plates with kanamycin 100 mg/L, rifampicin 50 mg/L, streaking was done by taking single colony per plate and grown at 28^{o}C for 48 h. Plasmids pNAV6051 and pNAV6052 were subcultured in YEP media with rifampicin 50 mg/L, gentamycin 50 mg/L. For agroinfiltration a single colony from each of the cultures containing pNAV6051, pNAV6052 and pCAMBIA 1305.2 were inoculated into 250 mL flask with YEB media (0.75% Bacto yeast extract, 0.8% nutrients Nutirent broth, pH 7.5) containing their respective antibiotics and the culture grown at 28^{o}C in a shaker with a 300 rpm, until the OD_{600} value was 1.7-2.0. The cultures were then spun at 5,000 g for 10 min then resuspended with agrosuspension medium (10 mM MES, pH 5.5; 10mM $MgSO_4$) and diluted to make the final OD_{600} vale upto 0.12.

2.5 Agroinfiltration

For syringe infiltration, 7-8-week-old plants were chosen with 5 leaves each per plant. The first three leaves counting from top of each plant were infiltrated with respective

A. tumefaciens cultures supension prepared as above. The fourth leaves were infiltrated with negative control (Ag1 culture without the vector). During infiltration a small nick was created with a needle in the abaxial epidermis (Yamamoto-Katou *et al.,* 2006). The infiltration was made by gently holding the leaf while applying the counter pressure to the nick with the thumb on one hand and injecting the infiltration solution into the nick with a syringe (Figure 3.2 a)

2.6 Bacterial strain used as a host for plastid transformation vector

Escherichia coli DH5α strain [F-φ80*lac*ZΔM15 Δ. (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-] (Invitrogen, Carlsbad, CA) was used as host for *gfp* and *cbhI* plastid transformation vectors.

2.7 Generation of the GFP expression vector

For cloning of expression vector primer designed contained *NcoI* and *EcoRI* sites in either end of the *gfp* fragments and expression cassettes ordered from GeneArt® such as 1007633_*Prrn-rps16*_pMAT, 1007634_*Prrn-psbA*_pMA-T, 1007635_*Prrn*(K)-*rps16*_pMA-T and 1007636_*Prrn*(K)-*psbA*_pMA-T. The resulting GFP expression

vector products were named as; pNAV201, pNAV202, pNAV203 and pNAV204 that were transformed in chemically competent *E. coli* cells. GFP fluorescence was detected in *E. coli* containing the above the GFP constructs. The pNAV55 is the empty vector with *aadA* cassettes and contains *NotI* and *HindIII* restriction sites for cloning purpose. The above GFP expression cassettes were inserted into the pNAV55 vector to obtain plastid homology vector and resulting vector was named as pNAV205, pNAV206, pNAV207, pNAV208.

2.8 Design of *cbhI* expression cassettes for CBHI.

The CBHI expression vector was cloned from GeneArt® synthesized expression cassettes. The full length *cbhI* were codon optimised with tobacco plastids expression ordered from GeneArt®. Both the vector and insert contained *NcoI* and *EcoRI* sites, and synthetic verson of CBHplas gene (0912925_CBHplas_pMK-RQ) obtained from GeneArt® and other expression cassettes such as; 1007633_*Prrn-rps16*_pMAT, 1007634_*Prrn-psbA*_pMA-T, 1007635_*Prrn*(K)-*rps16*_pMA-T and 1007636_*Prrn*(K)-*psbA*_pMA-T were digested with *NcoI* and EcoRI enzymes respectively. After digest with respective enzymes vector backbone and CBHI plas insert were gel isolated and ligation reactions were carried out with respective vector backbone fragments. The resulting product was named as pNAV222, pNAV223, pNAV224, pNAV225 and the sequencing of results were analysed for integration of the gene fragments (detailed information about the cloning of the above vectors can be obtained from Kim Stevenson Plant Biotechnology laboratory RMIT University).

The binary vector pCAMBIA13035.2 (12,361bp)www.cambia.org/daisy/bios/585.html contains GUS expression system. Binary vector with *cbhI* catalytic domain

pNAV6051 and pNAV6052 transformed in *A. tumefaciens* were obtained from Dr. Greg Nugent RMIT University.

2.9 Polymerase Chain Reaction (PCR)

For the amplification of the gene fragments by Polymerase Chain Reaction (PCR) Express Thermal Cycler (ThermoHybaid, Middlesex, UK) or a GeneAmp 2400 (PerkinElmer, Waltham, MA) were used. DNA amplification was carried out with using GoTaq Green Master Mix (Promega) using Promega standard protocols. For amplifying fragments larger than 4 kb the Long-Range PCR Kit (Life Technology) was used. Total PCR volumes were made up to 25 or 50 µL. Unless otherwise stated, the standard PCR protocol was followed: For 25 µL reactions, PCR components: Gotaq 12.5 µL, forward primer 1 µL primer reverse 1 µL, sterile milliQ H_2O 9.5 µL and template DNA 1 µL. The PCR products were stored at 4^oC.

Screening of the positive bacterium colonies for the presence of insert was conducted by inoculating the isolated bacterial colony with a sterile toothpick and resuspending the cells, in 10 µL sterile water in a PCR tube. For the confirmation of the plasmid vectors, 1 µL of the bacterial suspension were taken from each tube and amplifying using suitable primers at their desired product The PCR positive tubes were subcultured and maintained in a separate plate.

Screening of transplastidic tobacco lines (*gfp, cbhI* and *co14)* were performed by taking genomic DNA extracts and amplifying with gene specific primers. To determine longer fragment integration of the total vector in the tobacco plastid genome PCR with specific primers were carried out on the genomic DNA using

GoTaq Green Master 52 Mix (Promega) with initial denaturation step at 95^{o}C for 5 min, then 25 cycles of each cycle consisting of 94^{o}C for 30 s (denaturing), 55^{o}C for 30 s (annealing) and 72^{o}C for 3 min (extension) steps and a final extension at 72^{o}C for 7 min.

2.10 Preparation of bombardment experiment

2.10.1 Preparation of gold particles

Preparation and coating of gold particles were done according to a plastid transformation protocol with some modifications *(Verma et al.,* 2008). Fifty milligram of gold particles (0.6 µm) were prepared in 1 mL of molecular grade 100% ethanol and vortexed for 2 min and then centrifuged at 10,000 g for 3 min. To the gold pellet 1 mL of 70% v/v ethanol was added and the tube vortexed for 1 min and the mixture was incubated at room temperature (25^{o}C) for 15 min and mixed by gentle shaking and then spun at 5,000 *g* for 2 min. Gold pellet collected after discarding the supernatant was washed four times in 1 mL dH_2O, incubated at room temperature and spun at 5,000 g for 2 min. Final steps of the gold particle was resupended in 1 mL of 50% v/v glycerol and stored at -20^{o}C.

2.10.2 Coating of gold particles with DNA

Fifty microlitre aliquots of prepared gold particle from the stock solution were transferred into 1.5 mL microcentrifuge tube. While vortexing, 5 µg of plasmid DNA (1 µg/µL) was added, then 50 µL of 2.5 M $CaCl_2$, 20 µL of 0.1 M spermidine were added. Tubes were vortexed for 20 min at 4^{o}C. The DNA-coated gold particles were spun at 10,000 g for 1 min and the supernatant was removed. The pellet was washed with 200 µL of 70% v/v ethanol followed by 100% ethanol. The gold particle

coated with DNA was finally resuspended in 50 µL 100% ethanol and stored on ice for 2-3 h until used.

2.11 Particle bombardment of tobacco leaves

For bombardment, a PDS-1000/He Biolistic particle delivery system (Bio-Rad, cat. no. 165-2257), 0.6 µ gold microcarriers (Bio-Rad, cat. no. 165-2262) macrocarrier (Bio-Rad, cat. no. 165-2335), stopping screens (Bio-Rad, cat. no. 165-2336), 1,100 psi rupture discs (Biorad, cat. no. 165-2329) were assembled. About 8 µL of DNA coated gold particles was loaded onto the sterile macrocarrier placed in holder and proceed with bombardment using standard protocol (Verma *et al.,* 2008). After the bombardment, leaf plates were sealed with parafilm and kept in dark for 2 d and then each of the leaf was cut in 5 mm^2 pieces and placed on RMOP selection medium containing selection antibiotics as a selection agent for the shoot regeneration.

2.12 Confirmation of transgene integration in putative transformed spectinomycin resistant shoots.

Genomic DNA was extracted from 100 mg of leaf using DNeasy Plant Mini Kit - QIAGEN protocol for confirmation of gene integration. Gene specific primers for *aadA, cbhI*, *gfp* and *co14* were used in PCR. After the plants have been confimed for transgene integration by PCR, they were subjected to two further rounds of regeneration on medium with 500 mg/L spectinomycin dihydrochloride (Svab and Maliga, 1993). The putative transgenic shoots were maintained on plastic tub containing agar-solidified MSO medium containing 3% sucrose (Murashige and Skoog, 1962).

2.13 Southern blot gel preparation

2.13.1 Southern blot protocol

The genomic DNA for Southern blot analysis was isolated from the non-transformed and putative transformed shoots using the CTAB method. For the genomic DNA digest 10 µg of DNA was added into the reaction mixture (50 U of restriction enzymes, 1 mM spermidine, 1 % BSA, 1/10th NEbuffer and nuclease free distilled water added up to in 50 µL). To confirm the complete digestion 1/10th volume of the DNA digest samples were run on the 1% agrose gel and electrophoresis of genomic DNA was thus obtained. After confirming the complete digestion of genomic DNA at their restriction sites the DNA fragment were precipitated by taking 1/10th volume of 3 M sodium acetate (pH 5.2), 2 volumes of 100% ethanol added to the residual DNA digest. The mixture was then kept at -20^{o}C for 20 min to precipitate the DNA at bottom of the tube, spun at 10,000 rpm for 10 minutes, washed 2 times with 70% ethanol and the DNA was resuspended in 20 µL TE buffer. For the positive control, the *Bgl* II digested plasmid DNA sample was used. The molecular marker consists of 5 µL of λ DNA-HindIII digest/NEB. Agarose gel was prepared by boiling the mixture of 0.8% agarose gel in 100mL 1X TAE buffer. At 50^{o}C, 10 µg/mL of ethidium bromide solution (40 mM Tris-actetate, 1 mM EDTA) were added in the gel before pouring the mixture in gel tank. Load the digested DNA test samples with 5 µL of λ DNA-HindIII digest/NEB in the gel and run the gel at 35 V for 8-10 h for separation of the digested products. The gel photos were taken using a Biorad gel doc system. The gel was depurinated with 0.25 M HCl for 15 min, and equilibiated with 0.4 M NaOH for 20 min and transferred on to (Hybound-N+) (GE Health Bio-Science,

Rydalmere, NSW, Australia). Southern sandwich was made using the filter paper and tissue paper. The solution of 0.4 M NaOH was used for transfer of DNA from gel to the nylon membrane. The flat object about 500 g was kept above the sandwitch for equal distribution of pressure on the surface of the membrane.

2.13.2 Denhardt's hybridisation solution

Denhardt's hybridisation solution containing 50% Formamide, 4× SSC (2× SSC is 0.3 M NaCl plus 0.03 M sodium citrate), 5X Dehnardt's solution (Eppendrof cat# 0032 007.155), 5% dextran sulphate and 0.5% SDS (Wahl *et al.,* 1979). Denhardt's hybridisation solution was heated at 42^{o}C and 0.1 mg/mL fish sperm DNA was added to 20 mL hybridisation solution and heated at 95^{o}C for 5 min and snap cooled on ice for 5 min. The prehybridiastion was set up by placing the membrane in a hybridisation tube and adding 20 mL of hybridisation buffer and incubated for 2 h in a rotating oven at 42^{o}C.

2.13.3. Radiolabeling DNA probe using DecaPrimer II Kit

The Ambion® DECAprime™ II Random Primed DNA Labeling Kit was used for radiolabelling Southern blot probes. The reaction was performed using random decamer (10-mer) oligonucleotide primers and exonuclease-free Klenow enzyme (Exo–Klenow). DECAprime II Kit in used this experiment was according to the random primer labelling method (Feinberg and Vogelstein, 1983). The mix of decamer solution was hybridised with DNA template followed by heating at 100^{o}C for 10 min, and then by freezing. The complementary strand was synthesized from the 3' hydroxyl termini of random decamer primers by adding buffer, the nucleotides

(three non-labelled, one radio-labelled) and Exo–Klenow to remove all exonuclease activity (Derbyshire *et al.,* 1988).

The steps involved in the probe labelling were as follows. First $\alpha^{32}P$ was taken from freezer and placed behind Perspex screen to thaw. About 25 ng of DNA (dissolved in water or TE) was mixed to a final volume of 11.5 µL. Then 2.5 µL of 10X Decamer Solution was added and the mixture was incubated in boiling water for 5 min and then kept in ice for 3 min. The mix was then spot spun at top speed and kept on ice. After adding 5 µL of reaction buffer (-dCTP) – on the bench, the set up was moved to a hotlab to add 5 µL [$\alpha^{32}P$] dCTP and 1 µL Exo-Klenow. The reaction mix was gently mixed and quickly spun and incubated for 15 min at 37^{o}C followed by addition of 5 µL of of 2.4 M EDTA to stop the reaction.

2.13.4 Sephadex column preparation

To preapare a G-50 Sephadex column, some glass wool was put into a 3-cc syringe, and stuffed tightly with the plunger and loaded with 100 µL STE buffer (Sephadex pG-50, Pharmacia Biotech, Sweden). After 4 min the column was spun at 1000 g and this process was repeated until the Sephadex volume was up to 1 cm below the mouth. Then 25 µL labelled probe were added to 75 µL STE buffer, and the mixture was loaded to Sephadex column. The collection tube (1.5 mL) was placed inside 15 mL tube and the Sephadex column placed over the collection tube and entire set up was again placed over the 50mL tube. All the 50 mL tubes prepared similarly were spun for 4 min at 1000 g and purified probe sample was collected in 1.5 mL tube place at the bottom.

2.13.5 Preparation of probe for Southern blot

For Southern blot experiments *gfp*, *16S rRNA, cbhI* and *co14* DNA probes were made using designed primers (Table 2.1). PCR cycles included 2 min at 95^{o}C, 25 cycles of 30 s at 95^{o}C, 1.30 min at 72^{o}C and 5 min at 72^{o}C, followed by 5 min at 72^{o}C. The sizes of the probes were 1.3 kb, 1.115 kb, 1.34 kb and 1.32 kb for *gfp*, *16S rRNA, cbhI* and *co14* respectively.

Table 2.1 Primers for Southern blot probe

Primer Name	**Primer sequence**	**Melting temperature**
16S rRNA Forward primer	ATG GAG AGT TCG ATC CTG GC	54^{o}C
16S rRNA reverse primer	GCT TAT CAC CGG CAG TCT TGT	54^{o}C
cbh 18 forward primer	TAA CCA TGG CTC GTG CTC AAT CTG CTT	60^{o}C
cbh 19 reverse primer	TAC GTC GAC TTA ATT TCC ACC AGA AGG GTT.	56^{o}C
co14-Forward primer	AAA GGA TCC AAC ACT CAA GAA ACA CAC CC	60^{o}C
co14 reverse primer	AAA GTC GAC CTA TTT ACT ATA GGT TGA ATC	62^{o}C
gfp-f	TTA CTT GTA CAG CTC GTC CA	50^{o}C
gfp-r	TGA AGT TCA TCT GCA CCA CC	52^{o}C

2.13.6 Probe hybridisation

The membrane was placed in hybridization bottle filled with prehybridised solution and incubated for an hour and then replaced with hybridisation solution (pre-warmed 42^{o}C) incubated at 42^{o}C overnight. After discarding the hydridisation solution, the membrane was washed with wash solution 1 (2XSSC and 0.1% SDS) for 10 min and then washed three times at 5 min each with wash solution II (0.1% SSC and 0.1% SDS). The membrane counts were checked by a handheld Geiger counter and washed again in solution II at 68^{o}C till the minimal counts were observed.

2.14 Isolation of Total Soluble Protein (TSP) from chloroplasts

All the steps of the chloroplast isolation were carried out at 2-4^{o}C. Firstly 5 g deveined tobacco leaves were taken in 50 mL tube and 30 mL of 1X Chloroplast Isolation Buffer (CIB) with BSA **(**2 mM Na_2-EDTA**,** 1mM $MgCl_2$**,** 1 mM $MnCl_2$**,** 50 mM Hepes-KOH pH 7.5**,** 330 mM sorbitol**,** 5 mM sodium ascorbate and 1% BSA) (To *et al.,* 1996) was added. Then the leaves were homogenized with a polytron for 5 minutes and the mixture was filtered through a double layer of Miracloth™ into a prechilled 50 mL tube. The filtrate was then spun at 200 X g for 3 min. The supernatant was then transferred into another prechilled 50 mL tube and spun at 1000 X g for 7 min in a spin bucket centrifuge. A dark green layer in the middle was visible. Supernatant was removed gently and 1-2 mL of CIB (with 1% BSA) was

added and the chloroplasts were gently resuspended. Then chloroplast suspension was added on top of a 40% Percoll™ (6 mL of percoll and 4 mL of CIB with 1% BSA) (10 mL of 40% percoll for every 6 mL of chloroplast suspension). The mixture was then spun at 1700 X g for 6 min and middle green layer was collected after removing the upper clear interphase. The green pellet obtained was washed with CIB for 2 times to remove the excess percoll and final pellet was suspended in electroporation buffer (600 mM mannitol, 5 mM MES pH5.7, 15 mM KCl, 600 mM mannitol, 5 mM MES pH 5.7, 10 mM KCl) and the chloroplast number was estimated by using a haemocytometer. Chloroplast pellets (using 800 µl of $1x10^6$ chloroplast) were dissolved in 100 µl of extraction buffer (350 mm HEPES-KOH, pH 8.0, 10 mm $MgCl_2$, 5 mm EDTA, 14 mm β-mercaptoethanol, 3% [w/v] PVP 25, 15% [w/v] PEG 20,000, and 2.5% [v/v] Tween20™) to extract the TSP from isolated chloroplast (Maxwell, 1999). After treating with the extraction buffer the chloroplasts were snap frozen with liquid nitrogen and heated in 50^{o}C water bath for 5 times and vortex and grinded with pistol in each step.

2.14.1 Chloroplast electroporation

Electroporation of isolated chloroplasts was conducted using a mixture of 800 µl of 10^6 chloroplast, 4 mL dNTPs and 20 µg of DNA in 1.5 mL cuvette and electroporated with apparatus setting 250 µF; Voltage 400V; time 15 millisec (To *et al.,* 1996). During the electroporation the sample was suspended with culture buffer (600 mM mannitol 5 mM MES pH 5.7, 10 mM KCl) and kept at 25^{o}C for 24 h.

2.15 Microscopy

Leaf epidermal peels were prepared and mounted in mounting medium (IM030 Fluoro-gel, water based mounting medium with Tris-buffer from PST ProSciTech). GFP transformed chloroplast in guard cells were observed using Leica DM 2500 compound microscope and confocal microscopy.

2.15.1 Confocal microscopy

The epidermal peel from non-transformed and the putative transformed GFP plants were observed using the laser a scanning confocal microscopy facility at RMIT University Bundoora. Images were captured with excitation wavelength set at 488 nm and emission at 568 nm collected for FITC (Hibberd *et al.,* 1998). The images obtained with 100X magnification, 512X512 pixel resolution and 24-bit depth with scan speed of 9.

2.15.2 Electron microscopy

TEM experiment was conducted with Dr Chaitali Dekiwadiya from RMIT microscopy and microanalysis facility (RMMF). The fixative was prepared from 2.5% glutaraldehyde and 2.5% paraformaldehyde in a seawater-sodium cacodylate buffer. The wash buffer was prepared by dissolving 2 g of sodium cacodylate in 50 mL of distilled water and volume was brought up to 100 mL by adding autoclaved and then filtered sea-water at pH 6.8. Fixative was made by mixing stock neutralised glutaraldehyde (25% solution) and paraformaldehydes (25% solution) so that the final concentration of each solution to be 2.5%.

The leaf materials cut into 1 mm X 1 mm and fixed for 1-2 h at room temperature. After thorough washing with buffer the explants were post fixed in 1% OsO_4 solution buffer for 1 h. The explants were then dehydrated in graded solution of 50%, 75%, 80%, 90%, 95% and 100% of ethanol and then washed with acetone. The explants were resin embedded, sectioned with microtome and viewed in Jeol- 1010 transmission electron microscope imaging conditions 80Kv for plant samples.

2.16 Genomic DNA isolation from tobacco leaves

2.16.1 CTAB method for tobacco genomic DNA isolation

Genomic DNA from non-transformed and transgenic tobacco leaf tissue was isolated using a CTAB method (Gawel and Jarret, 1991). Tobacco leaves were pre-chilled with liquid nitrogen and ground with a mortar and pestle. To the powder, 10 mL of preheated CTAB extraction buffer [100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 3% CTAB (w/v) and 1% (v/v) of β-mercaptoethanol] was added. The mixture was transferred into a 50 mL tube, incubated at 65^{o}C for 30 min followed by the addition of 10 mL chloroform: isoamyl alcohol (24:1) mixture and incubated for 15 min at room temperature. After spinning for 5 min at 5000 xg at room temperature, the aqueous phase was filtered through miracloth to remove the cellular debris and an equal volume of ice-cold isopropanol was added to precipitate the DNA. The DNA was collected by centrifugation at 12,000 xg for 10 min at 4^{o}C and the pellet was washed with 70% ethanol, dried and re-suspended in 250 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). RNA was removed by adding 5 µl of 10 mg/mL RNaseA (Qiagen) and incubating at 37^{o}C for 30 min. DNA was precipitated by the addition of 1/10 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of 100% ethanol and the mixture was incubated at -20^{o}C overnight. The resulting mixture was spun at 13,000 xg for 10 min and DNA pellet washed with 500 µl of 70% ethanol then again spun at 10,000

xg for 3 min. Finally, the supernatant was discarded, DNA pellet was air dried and dissolved in TE buffer.

2.16.2 Frey method for genomic DNA extraction for PCR analysis

This method was used for rapid extraction of genomic DNA by taking 1 g of leaf material ground in 2 mL of lysis buffer (20 mM Tris, pH 8.0; 20 mM of EDTA; 2 M NaCl) (Frey, 1999). The mixture was incubated at 85^{o}C for 5 min, and then chilled on ice for 5 min. This process was repeated for 3 times. Finally, the tubes were vortexed and spun at 10,000 xg for 10 min. The supernatant was collected into a new tube and 5 µl of RNaseA was added (10 mg/mL) (Qiagen), then the mixture was incubated at 37^{o}C for 30 min. After the incubation, DNA was precipitated by adding 1/10 of the volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol, kept at -20^{o}C for 1 h. The resulting mixture was spun at 13,000 xg for 10 min and DNA pellet washed with 500 µl of 70% ethanol then again spun at 10,000 xg for 3 min. Finally, the supernatant was discarded, DNA pellet was air dried and dissolved in TE buffer.

2.17 Reverse transcription- PCR (RT-PCR)

For RT-PCR analysis preparation of total plant RNA made from 100 mg of leaf samples (Murashige and Skoog, 1962). Total cellular RNA was prepared following RNeasy plant Mini kit protocol (Qiagen) with slight modification. The leaves crushed using liquid nitrogen, followed by addition of the reagent mixture (Trizol reagent and RLT buffer at a ration of 1:1). After lysis reaction, the samples were centrifuged through a QIAshredder homogenizer. Ethanol was added to the cleared lysate. The

sample was then applied to the RNeasy Mini spin column and RNA was eluted in RNase-free water.

For RT-PCR analysis, cDNA was prepared using the 1 µg of RNA from respective transgenic samples and non-transformed leaf samples. The primers used for the cDNA preparation were the house keeping genes primers and gene specific primers listed in Table 2.2.

Table 2.2: RT-PCR primer

Primer Name	Primer sequence	Melting temperature
Tobacco Actin-F	TCA CTG AAG CAC CTC TTA ACC	52^{o}C
Tobacco Actin-R	CAG CTT CCA TTC CAA TCA TTG	50^{o}C
RT rbcL For	AAC AAG ATC GAA GTC GCG GT	52^{o}C
RT rbcL rev	GCT AGT TCC GGG CTC CAT TT	54^{o}C
RT psbA-B for	AGA GAC GCG AAA GCG AAA G	51^{o}C
RT psbA-B	CTG GAG GAG CAG CAA TGA A	51^{o}C
RT psaA For	CCC ATC TCA ACT GGG CAT GT	54^{o}C
RT psaA rev	ACG GGA ACT GCG AGC AAA TA	52^{o}C
cbh 18	TAA CCA TGG CTC GTG CTC AAT CTG CTT	60^{o}C
cbh 19	TAC GTC GAC TTA ATT TCC ACC AGA AGG GTT	-56^{o}C
ndh For	AAT GCA CGG TGT TCT TCG AC	52^{o}C
ndh Rev	TGC CTA ATT GTT CGG GTC CA	52^{o}C

2.18 Western blotting

For western blot analysis about 200-1000 mg of leaf tissue samples were snap frozen in liquid nitrogen and ground with a mortar and pestle to fine powder with ice cold protein extraction buffer (60 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM EDTA, 30 mM β-mercaptoethanol, 0.1 mM PMSF) or 50 mM sodium acetate pH 5.5 buffer in a ratio of 2 volume buffer to leaf weight. The mixtures were then transferred to 2 mL centrifuge tubes and spun at 10,000 xg at 4^{o}C for 4 min. The supernatant contained total soluble protein (TSP) which was quantified using the Bio-Rad Protein Assay (Bio-Rad, USA) protocol. For the western blot analysis 10-20 µg TSP extract was mixed with LDS buffer (Invitrogen) and boiled for 5 min. Protein were separated by electrophoresis on 12% ClearPAGE Precast gels for SDS-PAGE (C.B.S. Scientific). The protein marker used was Bio-line Hyper-Page Protein Marker (CAT No; bio-33065). The protein was transblotted on nitrocellulose membranes using iBlot™ Gel Transfer Device (Invitrogen). For the detection of the recombinant protein ONE-HOUR Western™ Detection System protocol (Llarrull *et al.,* 2011) with primary antibody (GFP/CBHI) and anti-mouse/anti-rabbit raised secondary antibody was used. The blot was rinsed twice in TBST for 5 min and added chemo-luminance substrate provided (from ONE-HOUR Western™ Detection kit) and bands were detected by placing the blot in between the two acetate sheets and exposing to Amersham Hyperfilm ECL film in developer and fixer for 30 s to 5-15 mins.

2.19 Activity assay

For detection of CBHI activity a MUC (4 methyl-Umbelliferyl–β-D-cellobiopyranoside) (Dai *et al.,* 2000a) assay was used. In this method 50 mM (i.e. 50 nmol/µL) of 4-MU

standard solution was diluted to 1 nmol/uL with water to make standard solutions ranging from 0, 2, 4, 8 16, 32 nmol. To same wells, sodium acetate (50 mM and pH 5.0) was added to bring up the volume of each tube at 40 µL. For the MUC positive sample 20 µL NaOAc (50 mM, pH 5.0) was added to each well along with 20 µL of respective samples, so total volume becomes 40 µL (these samples will receive (+) MUC reaction buffer) and a duplicate row of the above samples was created for MUC negative samples (these samples will receive (-) MUC reaction buffer). Then 100 µL of (-) MUC reaction buffer was added to 4-MU standards and 100 µL of (+) MUC reaction buffer added to (+) MUC set of samples. Then 100 µL (-) MUC reaction buffer was added in (-) MUC set of samples. After this set up the 96 well plate was covered and wrapped in aluminium foil and incuabated at 55^{o}C for 1 h and the reaction was stopped by adding 100 µL of 0.15 M glycine solution, pH 10 to each well and fluorescence was read using Bioline spectrophotometry.

2.19.1 Preparation of 50 mM 4-MU Stock solution

The weight of 4-methylumbelliferone (MW = 198.16) taken to 99.1 mg and dissolved in 10 mL MQ H2O containing 12.5 mg 4-methylumbelliferyl β D cellobioside (MW = 500.5) and dissolve in 10 mL MQ H2O. The prepared solution of 4-Methylumbelliferyl β D cellobioside wrapped in foil and stored at 4^{o}C, for up to 2 weeks. The reaction stop solution was prepared by weighing 1.126 g glycine (MW = 75.07) dissolved in 80 mL MQ H_2O (pH 10) first and bring up the volume upto 100 mL distilled H_2O. Simillary 100 mL of 50 mM and 100 mM Sodium acetate (pH 5.0) stock solution was also prepared.

2.20 E. coli culture medium

LB (Luria Bertani) contains (1% trypton, 0.5% yeast extracts, and 1% NaCl) and LB Lennox broth contains (1% trypton, 0.5% yeast extracts, and 0.5% NaCl.

2.21 Agarose gel preparation

To the melted Agarose/TAE gel (1% v/v) 1-5 µl of ethidium bromide at 50^{o}C was added and the mixture was poured into the gel casting tray with the comb.

2.22 Biorad protein assay

The TSP from bacterial or plants were estimated with the Bradford method (Bradford 1976). Protein standard from 0.625 to 20 µg were used to generate a standard curve in Greiner 96-well plate. Total volume in each replicate well was 220 µL. The mixture was incubated for 5-30 min and optical density was read at 595 nm.

Chapter 3: Methodology chapter to demonstrate cellobiohydrolase gene (*cbhI*) in tobacco leaves.

3.1 Introduction

In transient expression systems the recombinant gene does not integrate into the host genome after delivery, but is expressed by the host utilising the cellular transcriptional and translational machinery (Escudero and Hohn, 1997, Voinnet *et al.,* 2003). Methods used for transient expression are: agroinfiltration (Voinnet *et al.,* 2003), virus mediated transformation (Scholthof *et al.,* 1996), bombardment (Twell *et al.,* 1989), microinjection (Toyoda *et al.,* 1990), PEG (Spörlein et al., 1991) and electroporation (Ow et al., 1986) or protoplast fusion (Yoo *et al.,* 2007). Agroinfiltration is a rapid method which can take a few hours or up to 7 d for assaying transgene expression in plants (Janssen and Gardner, 1990, Kapila et al., 1997, Wroblewski *et al.,* 2005). These methods have been used for a wide range of applications such as; transgene complementation studies (Shao *et al.,* 2003), a promoter analysis, recombinant protein production (Yang *et al.,* 2000) and tissue localisation studies (Twell *et al.,* 1989).

Nicotiana benthamiana is commonly used for agroinfiltration because its leaves are easy to infiltrate and allow multiple assays to be conducted in a leaf (Chen and Lai, 2013). Use of a syringe allows the easy penetration of *Agrobacterium* suspension containing recombinant vector into the intercellular spaces of leaf (Vaghchhipawala *et al.,* 2011).

Steps involved in the introduction of a foreign gene into plant tissue by agroinfiltration are; plant growth, culture of *A. tumefaciens* containing the plasmid binary vector and

infiltration (Leuzinger *et al.,* 2013). The choice of fully expanded true leaves of *N. benthamiana* is important for agroinfiltration; for example young leaves from newly grown plants have higher expression of recombinant protein than medium and older aged leaves (Wroblewski *et al.,* 2005). The concentration of *Agrobacterium* used for agroinfiltration was at OD_{600} 0.3- 0.5 (Wroblewski *et al.,* 2005).

Transient expression of recombinant cellulases in plant using *Agrobacterium* vectors had been method of choice due its speed, high yield and versatility of expression. From the transient expression in *N. benthamiana* 12.5 % of CBHI from *T. reesei* were recovered (Hahn *et al.,* 2014).

The main purpose of work described in this chapter was to transiently express the *T. reesei cbhI* in *N. benthamiana* leaves to validate the use of an anti–CBHI antibody in CBHI detection and quantitiation. The cross-reacting band with CBHI-antibody at the predicted size from the TSP of transformed *N. benthamiana* would confirm the plant expression of CBHI.

In this chapter *Agrobacterium*, harbouring binary vector with expression cassettes containing the coding region of the catalytic domain of *cbhI* were infiltrated into tobacco leaves and cellobiohydrolase (CBHI) levels and activity were examined. The expression of the enzymatically active catalytic CBHI in *N. benthamina* has not been previously reported. This chapter reports the transient expression of the β-glucuronidase (GUS) reporter (Control) gene that confirms the method used is effective and transiently expressed *cbhI* in the tobacco cells leading to the accumulation of detectable cellulases enzyme 5 d post–infiltration. The expression cassettes contained the binary vector of leader peptide *apo*-fused with *cbhI* directing the recombinant protein expression into the apoplast (Figure 3.2).

3.2 Materials and methods

The steps involved in the agroinfiltration includes; plant material for transient assays, *Agrobacterium tumefaciens* culture prepation, agroinfiltration and protein assay were presented in Chapter 2 (2.3, 2.4, 2.5, 2.18 and 2.19). The agroinjection was performed by creating nick on the leaves surface and injecting the agrobacterium dissolved with infiltration buffer into the intercellular space of the leaf.

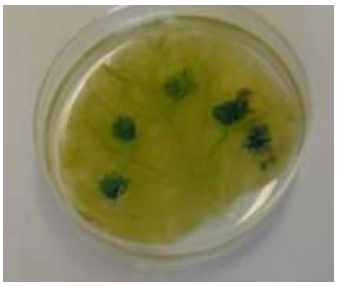
3.3 Results

3.3.1 GUS expression

Three days postagroinfiltration, the injected leaves were treated with the colourless X-gluc (5-bromo-4-chloro-3-indoyl-D-glucuronic acid) substrate, incubated overnight at 37^{o}C for development of blue spots in the agroinfiltrated sections of leaves. Plant leaves showed significant GUS staining at agroinjection sites and did not induce any chlorotic or necrotic responses (Figure 3.1). These results provided confidence in the use of the agroinjection methodology to detect *cbhI* expression in plants.

a. GUS expression cassettes

b. Agroinfiltration



c. GUS expression- blue spot

Figure 3.1 Gus expression system: a. GUS (β-glucuronidase) expression cassettes in binary vector pCAMBIA 1305.1 contains *gus* driven by cauliflower mosaic virus 35S (CaMV 35S) promoter and nopaline synthase (NOS) terminator. b. Syringe infiltration of *N. benthamiana* leaves with *A. tumefaciens* harbouring expression vector resuspended in infiltration buffer and loaded into a syringe c. blue spots represent GUS expressed leaf sample.

3.3.2. Transient expression of CBHI in *N. benthamiana*

The binary vector pNAV6051 contained the *cbhI* sequence fused with a signal peptide (*Apo'*) sequence that directed the section of CBHI into the apoplast space (Figure 3.2). The binary vector pNAV6052 contain *cbhI* expression sequence fused with signal peptide (Apo) and *PR1a* sequence known to direct the recombinant protein into the apoplast (Moloney and Holbrook, 1997). The expected molecular weight of the catalytic verson of *T. reesei* CBHI is 46 kDa. The expressed CBHI in the agroinfiltrated samples should be detected by immunoblot using anti-CBHI-antibody.

pNAV6051

pNAV6052

Figure 3.2 CBHI expression cassettes in binary vector under the regulation of *N. tabaccum* rbcS promoter and nopaline synthase (NOS) terminator. In pNAV6051 *rbcS* promoter with leader peptide *apo*'-fused with *cbhI* and there is absence of *PR1a* sequence in this construct. While pNAV6052 cassettes contain rbcS promoter with additional sequence of amino acids *PR1a* region fused with *apo-cbhI* that codes for a peptide to divert the CBHI to the apoplast via the secretary pathway.

3.3.3 Western Blot results

Figure 3.3 represents the western blot of the crude extracts from the first set of experiments where agroinfiltrated leaves (vectors pNAV6051 and pNAV6052) were harvested 3 d, 5 d, 7 d and 9 d post infiltration. The positive controls were full length CBHI (molecular Wt. 56 kDa) and catalytic CBHI (molecular Wt. 46 kDa) (Figure 3.3). There exists variation in expression of CBHI in expression from both the binary vectors.

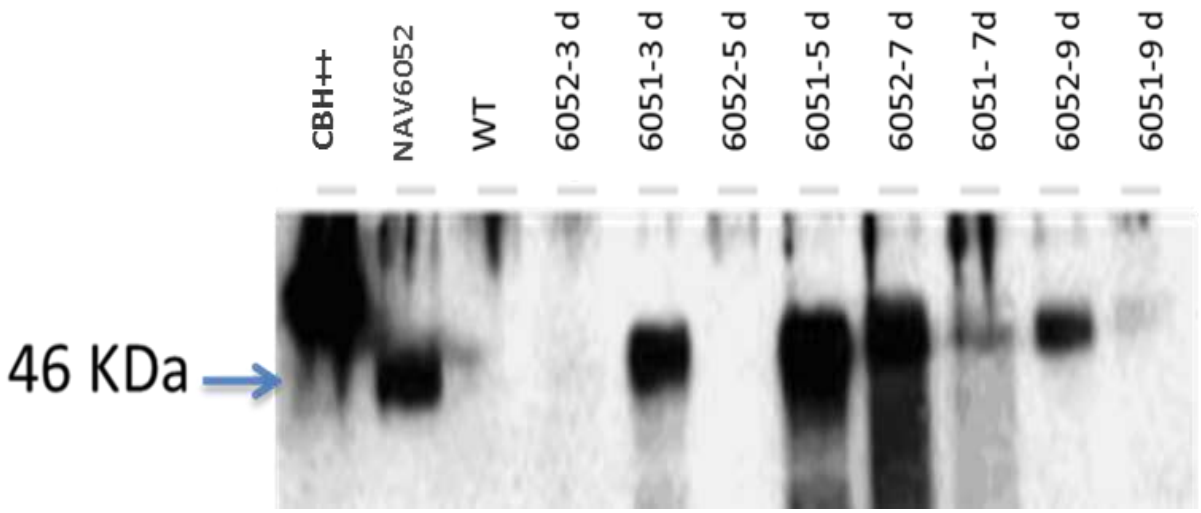


Figure 3.3 Immunoblot analysis of CBHI produced post agroinfiltration with pNAV6051/pNAV6052 in *N. benthamiana* leaves. First lane from left represents positive control (30 ng purified full length CBHI) with expected band at 56 kDa and second lane had extracts from pNAV6052 plant expressed with catalytic domain of CBHI. Cross reacting bands for the recombinant protein expected to migrate at 46 KDa. Since the promoter from the small subunit of rubisco (rbcS) that was used for the expression of the *cbhI* is the light dependent, the comparision was made to determine if any difference in level accumulation of recombinant protein was observed in samples harvested in morning or in evening. The plant used for this experiment was grown in controlled temperatue growth room under artificial light of 16 h light / 8 h dark photoperiod and 25^{o}C constant temperature.

A second agroinfiltration experiment was set up using *N. benthanmiana* leaves to investigate if the variation observed was due to light dependence when rbcS promoter drives the expression of CBHI. For each construct (pNAV5051 and pNAV6052), two *N. benthanmiana* plants were used, the four top leaves were infiltrated from each plant, leaves infiltrated at five spots on each leaf and TSP extracted from whole leaf, and loaded in the gel to obtain the western blot (Figure 3.4). The western blot results show that accumulation of CBHI was higher in the agroinfiltrated samples harvested at evening time from 7 d onwards (Figure 3.4). The rubisco content in the photosynthetic leaves increases during the day compared to that in the night (Russell *et al.,* 2013). Due to rubisco promoter used for driving the expression of the CBHI in the expression cassette limiting amount of CBHI synthesis

occured during night time (Figure 3.2). Thus, it was decided to harvest the leaves after 7 d during evening time for higher expression.

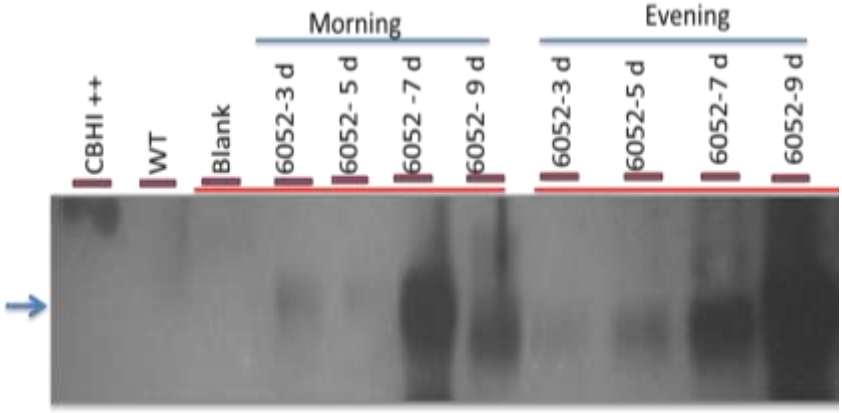


Figure 3.4 Immunoblot of CBHI expressions in *N. benthamiana*. Leaves were collected in the morning and in the evening at day 3, 5, 7 and 9 post-infiltrations.

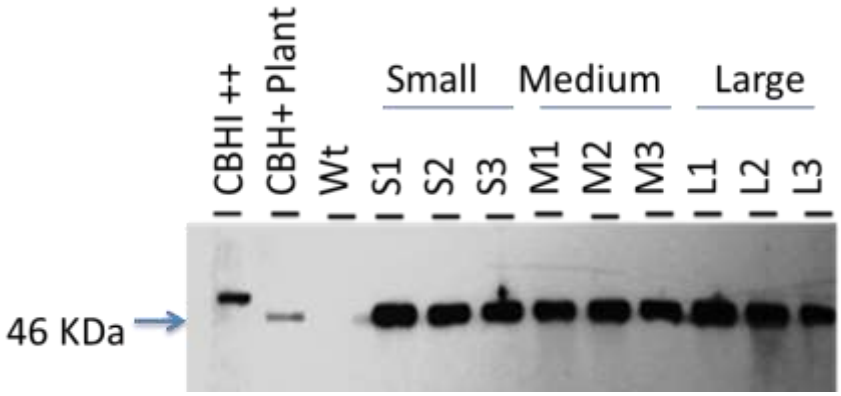


Figure 3.5 Immunoblot of *N. benthamiana* agroinfiltrated with binary vector pNAV6052 containing catalytic domain-*cbhI*. Positive controls include 30 ng of purified full length CBHI ++ and 10 µg of TSP from *N. benthamiana* expressed catalytic version CBHI (positive control obtained from Dr. Gregory Nugent RMIT University) with molecular weight 46 kDa, lanes S1, S2 and S3 agroinfiltration of small leaves, lanes M1, M2 and M3 agroinfiltration of medium leaves, lanes L1, L2 and L3 agroinfiltration of large leaves. Each lane contained 10 µg of leaf TSP. Note that the plants used in these experiments were grown in glasshouse conditions, the samples were harvested 7 d post-agroinfiltration.

A third experiment was set up by growing the *N. benthamiana* plants in a glasshouse with 25^{o}C day/night temperature and 16 h light/8 h-dark cycle. For this experiment 8-week-old plants, typically at the eight-leaf stage were used. In this experiment three *N. benthamiana* plants were used, three top leaves were infiltrated from each

plant with *A. tumefaciens* OD_{600} 0.3 and western blot results from the 7 d post-agroinfiltration of this extract showed an even expression of the CBHI gene within 9 samples that were harvested during evening time (Figure 3.5).

3.3.4 Enzymatic activity of CBHI in transient assays

Leaf extracts were tested for CBHI enzymatic activity using a fluorescence base assay with MUC substrate. CBHI activities for the evening harvested samples were higher than those of morning harvested samples (Figure 3.6). The CBHI activity remains highest in 5 d samples that were harvested in the evening. Variation existed in activity assay of the samples harvested from both the 5 d and 9 d samples (Figure 3.6). In general, the activity of samples harvested during evening was more than that of samples harvested in the morning, suggesting the presence of more CBHI that was synthesised during the day.

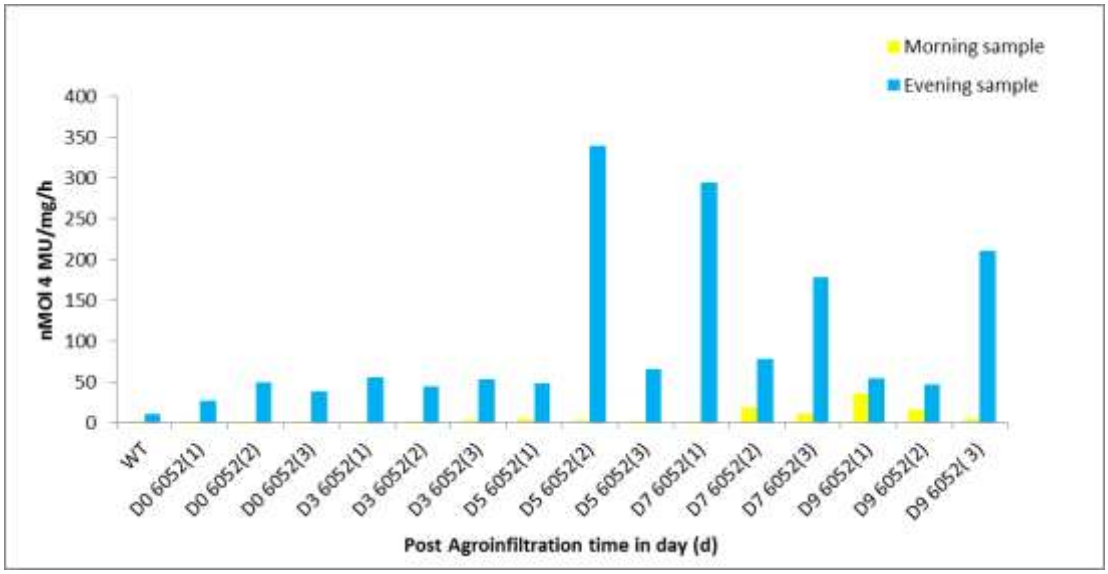


Figure 3.6 Activity assay for CBHI expressed in morning and evening samples in *N. benthamiana* and WT (non-transformed), 3 d, 5 d, 7 d and 9 d samples. The triplicates samples analysed for each day were from separate injections into three different leaves.

The results of the activity assay of agroinfiltration experiments from the glasshouse grown plants showed that top, smaller leaves showed fairly higher activity than both the medium and the larger sized leaves (Figure 3.7).

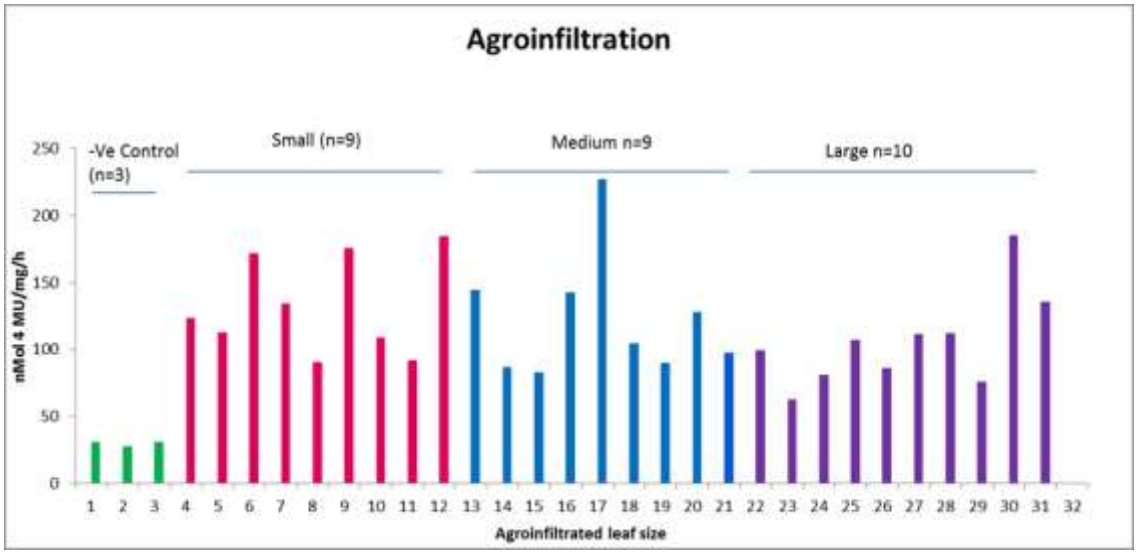


Figure 3.7 Activity of CBHI expressed in *N. benthamiana* glasshouse grown plants: Control: agroinfiltration of leaves with *Agrobacterium* Agl1 strain without transformation vector. At 7 d agroinfiltration with *cbhI* binary vector, *N. benthamiana* leaves were harvested. The leaves selected for the agroinfiltration were small (4-12), medium (13-21) and large (22-31). MUC activity expressed as nmol 4MU/mg TSP/h.

3.4 Discussion

The work reported in this chapter demonstrates that the syringe agroinfiltration method is a fast, reliable and low-cost method for transient expression of both the GUS and CBHI genes. The efficiency of the agroinfiltration and transient expression was confirmed from the appearance of blue spot for the GUS expression 3 days post agroinfiltration of *N. benthamiana* leaves with an *A. tumefaciens* harbouring a *gus* expression vector (Figure 3.2b). The validation of anti–CBHI antibody was confirmed by detection of CBHI in immunoblot of TSP extracts of *N. benthamiana* leaves post agroinfiltation (*A. tumefaciens* harbouring binary *cbhI* vector) (Figure 3.3).

In order to minimise the variation coming from unequal distribution of the CBHI expression level and its activity within an infiltrated agroinfiltrated leaves, samples were harvested in morning and evening to monitor the expression of CBHI as the light dependent rubisco promoter was used in the expression cassettes (Snir *et al.,* 2006). The harvest time of the leaves for maximum accumulation and higher activity was noted in evening time harvested sample (Figure 3.4). The western blot result showed gradual accumulation of CBHI as 3 d, 5 d and 7 d. There were higher total CBHI accumulation levels and activity when leaves were harvested during evening. Both of these observations are consistent with higher levels of rubisco found in leaves during evening as stomata closer commences from late afternoon (Paulsen and Bogorad, 1988). Maximum CBHI was recorded at 7 d samples (Figure 3.4). The expression level of CBHI among 9 different TSP extracts were estimated to be up to 2.5 % of total soluble protein (Figure 3.5) and its activity around 75-150 nMol 4-

MU/mg TSP/h (Figure 3.7). However, there were consistent cross-reacting bands of CBHI obtained from 8 weeks old healthy plants grown at standard glass house environment and 7 d post-agroinfiltration on the sample harvested during evening (Figure 3.5).

As observed previously (Van der Hoorn *et al.,* 2000), the necrosis response has a direct effect on the expressed gene products and this phenomenon was observed with collapsing of the plant tissue due to extensive cell death within the infiltrated portion (Klement and Goodman, 1967). A limitation of the agroinfiltration method lies in the formation of necrosis in the infiltrated leaves, which also depends on the virulence of the *A. tumefaciens* strain, plant physiological conditions, compatibility between the plant and the bacterium (Wroblewski *et al.,* 2005) and bacterial ability to encode T-DNA materials (*Nam et al.,* 1997). In this experiement necrosis was observed in leaves infiltrated with *Agrobacterium* suspension containing both pNAV6051 and pNAV6052 vectors only after 7 days of post agroinfiltration and despite its limitation this method was useful to detect expressed CBHI as early as 3 days of post agrofiltration and uptimal expression was achieved on 7d post infiltration. However, the agroinfiltration method was useful in early analysis and detection of expression of CBHI in plant cells within a few days' time rather than waiting up to months to develop the transgenic plants.

Chapter 4: Plastid transformation with GFP vectors

4.1 Introduction

4.1.1 Overview of plastid transformation

Plastids are semi-autonomous cellular organelles of plants and algae. Plastids are highly polyploid circular double stranded DNA, 120 -180 kb length and encoding about 120 genes (Wani *et al.,* 2010). Most higher plant plastid genomes contain a pair of large inverted repeats of approximately 25 kb in size (Maliga, 2004). The main functions of plastids in plants are; photosynthesis, starch accumulation and biosynthesis of some amino acids, lipids and pigments.

Plastid transformation offers a number of attractive advantages over nuclear genome transformation such as: high-level of transgene expression (Daniell *et al.,* 2002, Maliga, 2004, Purton, 2007), multigene expression in a single transformation event (Staub and Maliga, 1995, Gruissem and Tonkyn, 1993), transgene containment via maternal inheritance (Daniell *et al.,* 1998, Maliga, 2003), lack of gene silencing (Lee *et al.,* 2003) and position effects when transformation is mediated by site specific transgene integration (Daniell *et al.,* 2004). The main challenge of plastid transformation is the lack of adequate tissue culture systems for target crop species (Nugent *et al.,* 2006, Verma and Daniell, 2007). Plastid transformation was first reported in *Chlamydomonas reindhartii* (Boynton *et al.,* 1988), introduction of foreign DNA into plastid genome has been shown to be possible by methods such as; micro-projectile bombardment (Svab *et al.,* 1990), DNA electroporation (To *et al.,* 1996) and use of PEG-mediated treatment into the isolated protoplast (Dovzhenko, 1998).

4.1.2 Properties of GFP

Green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has been widely used as reporter protein in microbes, plants and animals. GFP is a thermo-stable protein that can be expressed in wide range of organisms *in vivo* or in cell culture. Purified GFP contains 238 amino acids which absorbs blue light maximally at 395 nm and exhibits a smaller absorbance peak at 470 nm (Cody *et al.,* 1993) and emits green light with peak emission at 509 nm (Morise *et al.,* 1974).

4.1.3 GFP detection

Green Fluoroescent Protein (GFP) expression was first demonstrated *in E. coli* (Chalfie et al., 1994), then in number of other organisms including yeast*; Caenorhabditis elegans, Drosophila* (Wang and Hazelrigg, 1994), mice (Okabe et al., 1997) and plants (Sheen et al., 1995). GFP was used to monitor gene expression and protein localisation studies in various heterologous systems (Chalfie et al., 1994, Haseloff and Amos, 1995). Both transient as well as stable transformation strategies have been used to target GFP expression in plants. For example, transient expression of GFP in plants through bombardment of tobacco leaves was useful to conveniently check the expression of a *gfp* vector (Hibberd et al., 1998). Stable transplastidic expression of GFP using *Prrn* promoter and *psbA* terminator in potato has been reported (Sidorov et al., 1999) followed by reports of the generation of several GFP transplastomic plants (Hibberd et al., 1998, Lelivelt et al., 2005, Yang et al., 1998). Plastid expression of GFP was also obtained by targeting functionality of ribo-switch harbouring expression cassettes (*Prrn*–*5′*UTR–*gfp*–*Trps16*) (Verhounig et al., 2010).

GFP expressed in plant cells after appropriate post translational cyclisation steps undergo folding and oxidation for its optimal function (Haseloff and Amos, 1995). GFP fluorescence can be directly detected using flow cytometry methods but the limitation of this technique is that it is pH dependent process and needed oxygen for the emiting fluorescence signals (Heim *et al.,* 1994). The localisation of GFP in excised plastid transformed leaf tissue detected by FACS (fluorescence-activated cell sorting) or CLSM (confocal laser scanning microscopy) techniques with the excitation at 488 nm and 635 nm for GFP and chlorophyll respectively (Cui *et al.,* 2014). Other techniques used for the GFP detection include whole plant UV illumination, epifluorescence microscopy and by electrophoresis (Santa Cruz, 1995).

There are two main uses of GFP in plants: (1) to monitor gene expression and protein localisation, (2) provide an easily scorable genetic marker in living cells (Haseloff and Amos, 1995). A significant advantage of GFP is that transformed tissue need not undergo cell lysis and tissue disruption as is the case with *β-glucuronidase* reporter gene requires staining (Chalfie *et al.,* 1994).

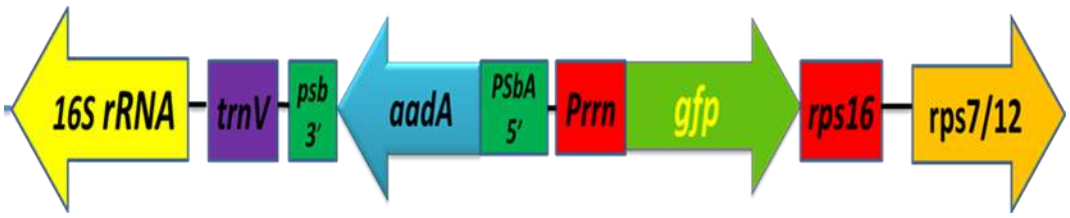


Figure 4.1 Map of the chloroplast expression vector shows the integration sites, *16S rRNA* and *rps7/12, aadA* selectable marker genes, and *gfp*.

This chapter aims to validate the plastid expression cassettes using *gfp* (Figure 4.1) which contain cellulases gene insert used for cellulases expression (Chapter 6). The integration of the *gfp* expression cassettes was targeted to the region between the *16S rRNA* and *rps12/7* loci of the tobacco genome (Michoux *et al.,* 2011). It had been reported that insertion of a translational enhancer sequence (k) at the N-terminus was found to yield highest expression levels (Herz *et al.,* 2005). The GFP expression vectors used are summarised in Table 4.1. The GFP was detected in *E. coli* cells containing plastid expression cassettes with *Prrn-gfp* (Verhounig *et al.,* 2010).

4.2 Materials and Methods

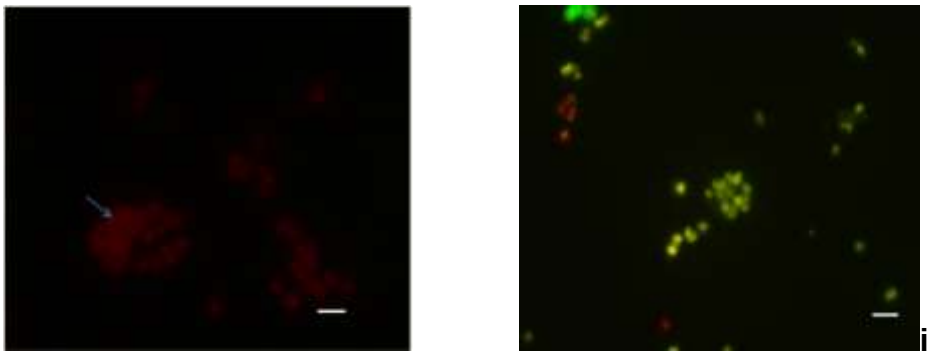
The methods used in this chapter such as isolation of chloroplast, electroporation of isolated chloroplasts, genomic DNA isolation, PCR, western blot, confocal microscopy, Southern blot and stable plastid transformation via homologous recombination have been described in Chapter (2). The *gfp* expression cassettes were constructed in our laboratory by Kim Stevenson.

4.3 Results

4.3.1 Transient expression of GFP in isolated chloroplasts

The transient expression assay enables to study of the function, regulation and localization of a gene within the chloroplast and complement the stable transformation over a short period of time. GFP fluorescence signals from

chloroplasts electroporated with pNAV204 vector were detected after 72 h using fluorescence microscope (Figure 4.2). When observed under fluorescence microscope, pNAV204 electroporated chloroplast samples produced greenish yellow fluorescence (signal from the expressed GFP) and non-transformed chloroplast was red colour (due to chlorophyll auto-fluorescence). This result validated the *gfp* expression cassettes by transient assay using electroporation of plasmid DNA into the chloroplast.



a. b.

Figure 4.2: Electroporated chloroplast examined using fluorescence microscope: a. Red spherical structure indicates non-transformed chloroplast. b. Fluorescent spherical structure indicates electroporated chloroplst with pNAV204 chloroplast vector expressing GFP. (Scale bars: 10 µm)

4.3.2 Integration of *gfp* into the plastid genome

Table 4.1 summarises the *gfp* expression cassettes, with total number of bombardments along with numbers of positive shoots recovered. The transformation frequency for pNAV205 vector was highest among pNAV207 and pNAV208 vectors (Table 4.1). However, there were no shoots obtained from pNAV206 plants.

Table 4.1 Plastid transformation of the GFP lines:

Plasmids	Total bombardments	Resistant shoots	Postive shoots	Expression cassettes
pNAV205	20	16	13	*Prrn-gfp-rps12*
pNAV206	40	1	0	*Prrn-gfp-psbA*
pNAV207	20	10	8	*Prrn(K)-gfp-rps12*
pNAV208	20	4	3	*Prrn(K)-gfp-psbA*

4.3.3 PCR analysis

For screening of putative transplastidic GFP plants, PCR using *gfp* primers on the plant genomic DNA extracts was conducted. The PCR results (Figure 4.3), confirmed 4 shoots from pNAV205 (lane 2-5), 4 shoots from pNAV205 (lane 6-9) and 3 shoots from pNAV208 (lane 10-12). The wild type (lane 13) lack the band as expected and lane 15 was the positive control used (pNAV205 plasmid) was at 1.3 kb.

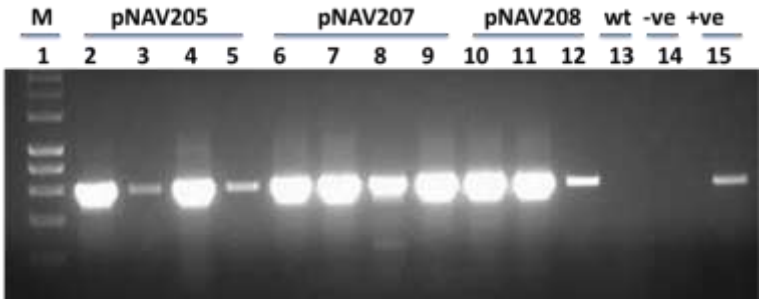


Figure 4.3: PCR results of stable transplastidic GFP plant with *gfp* specific primers lane 1 1 kb DNA ladder, lane 2 pNAV205-1, lane 3 pNAV205-2, lane 4 pNAV205-3, lane 5 pNAV205-8, lane 6 pNAV207-1, lane 7 pNAV207-3, lane 8 pNAV207-4, lane 9 pNAV207-5, lane 10 pNAV208-1, lane 11 pNAV208-2, lane 12 pNAV207-3, lane 13 wild type (non-transformed) and lane 14 no DNA (Negative control) and lane 15 positive control pNAV208 plasmid template.

4.3.4 Southern blot analysis

For Southern blot analysis a GFP probe was used for detecting the integration of the expression cassettes in the putative transformed spectinomycin resistant shoots. Figure 4.4 represents the map of expression cassettes showing the homology region and the integration sites within the chloroplast genome. A fragment of plant DNA of 6.8 kb was predicted to hybridise with GFP probe and no band in the non-transformed plant as expected.

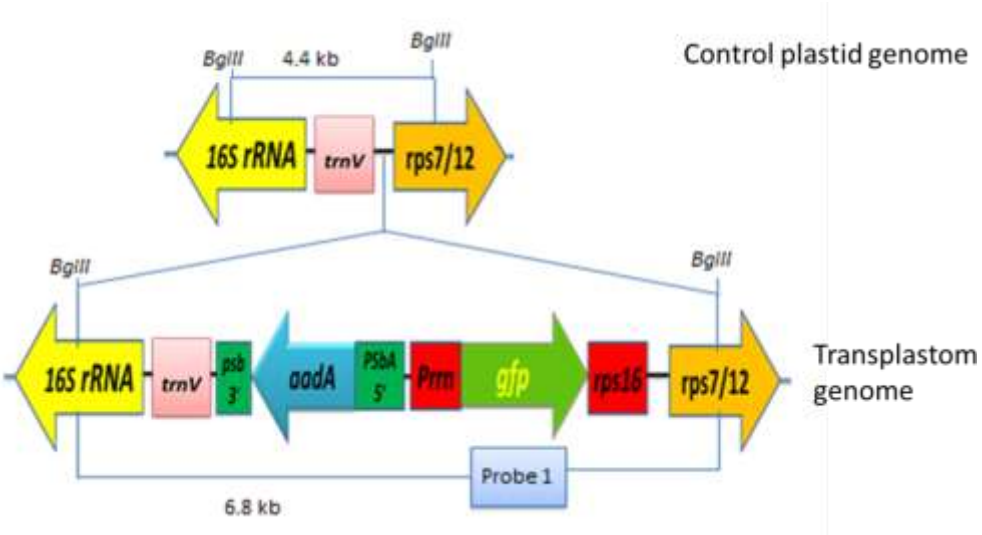


Figure 4.4 Map of wild type (non-transformed) and transplastomic GFP plant genomes showing the expected restriction digestion fragment detected by the Southern blot with gfp probes. *Bgl II* sites used for RFLP analysis of gDNA extracts were marked.

A Southern blot was set up with 2 out of 10 PCR positive shoots from pNAV205, 3 out of 8 positive shoots from pNAV207 and 2 out of 3 positive shoots from pNAV208 (Figure 4.5), showed that integration had occurred in respective plants bombarded with the three *gfp* plasmids (pNAV205, pNAV207 and pNAV208). The *gfp* probe hybridised and the band obtained was 6.8 kb aligning at the targeted location (*16S rRNA and rps7/12*). As expected there was no cross reacting bands for the non-transformed genomic DNA with the *gfp* probe.

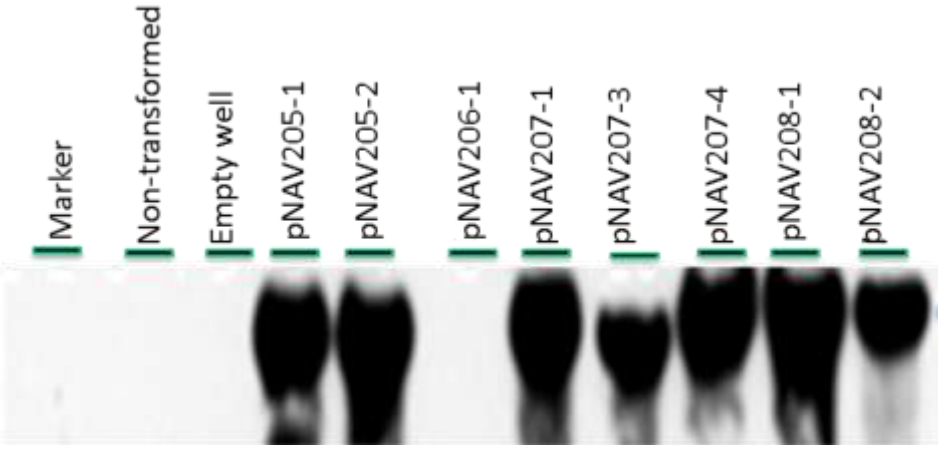


Figure 4.5: Southern blot analysis of the GFP transformed shoots: For the Southern blot DNA 10 µg of gDNA samples from each transplastomic plants were digested with *Bgl* II and hybridised with a radiolabelled probe *gfp* probe. The positive used was *Bgl* II digested plasmid DNA of pNAV205-1.

4.3.5 Western blot results for the GFP transformed shoots

The expression level of the GFP was confirmed with the western blot. From the western blot result it was evident that GFP expression of pNAV207 was higher than of pNAV205 which implies enriched GFP signal is due to the presence of 5 amino acid sequence (K) in pNAV207 expression vector. The amount of GFP (Figure 4.6) was more in pNAV208 than that of pNAV207 and pNAV205 lines. There was no band for spectinomycin resistant shoots (pNAV206). The positive band in the pNAV205-flower suggests that *gfp* sequences had been expressed even in the chromoplast.

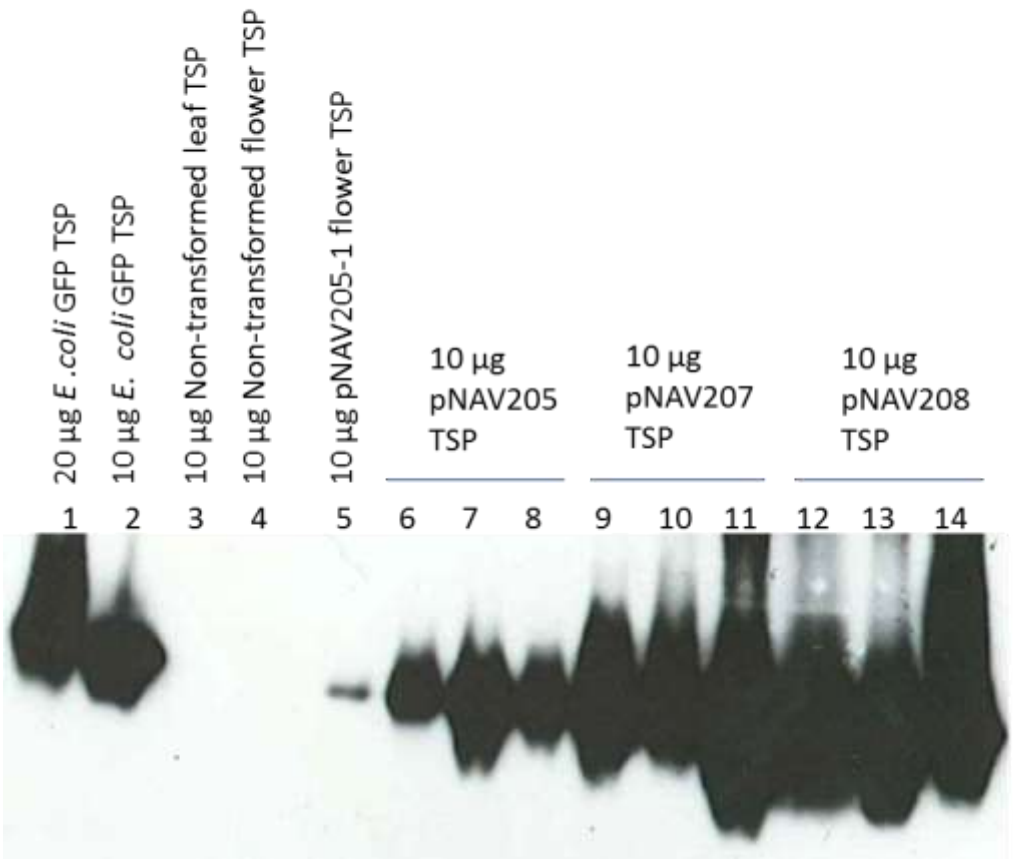
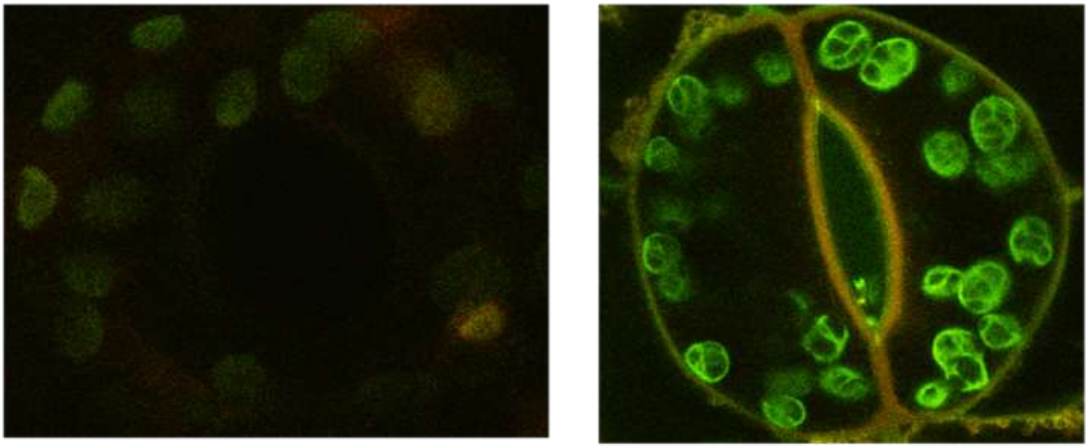


Figure 4.6 Immunoblot detection of GFP accumulation in transplastomic tobacco.

4.3.6 Detection of GFP fluorescence in transplastidic tobacco leaves using fluorescent microscopy

GFP transformed plants were also identified using confocal microscopy. Images obtained from non-transformed and pNAV205 and pNAV207 transplastidic plants are shown (Figure 4.6). The GFP was visualised in the guard cells chloroplast of pNAV205-1 when viewed through confocal microscope using FITC/TRIFC filters (Figure 4.7). The intensities of non-transformed plant were very weak as compared with GFP transformed plants.



A. Non-transformed leaf B. Guard cells of pNAV205 transplastidic plant

Figure 4.7: A. Confocal microscope image of the non-transformed type and transplatomic GFP plant guard cells. Image obtained using FITC/TRIFC filters.

4.3.7 Detection of TSP of GFP fluorescence in transformed plants using UV light

After passing UV light through TSP extracts from GFP expressed plants; green fluorescence was observed in TSP extracts of pNAV205-1 and pNAV207-1 plants and the non-transformed plant leaves were colourless (Figure 4.8 shows). Hence this result shows that expressed GFP remain stable in the TSP extracts.

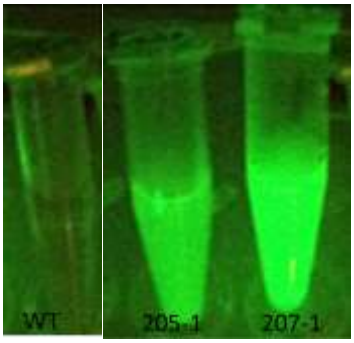


Figure 4.8: Figure 4.8 GFP fluorescence of TSP extracts of transplastomic plant (pNAV204-1 and pNAV207-1) and under UV light

4.4 Discussion

As expected GFP expression in *E. coli* was observed during routine propagation of plasmids (data not sown). The best expression of GFP was from pNAV208 transplastomic plants and the yield of pNAV207 was higher than pNAV205 which may be due to the enhancer sequence (K). There were no apparent phenotypic effects of GFP accumulation on the growth and development of transplastidic plants. All transformants grew normally on MS medium with 500 mg/L spectinomycin. The first-generation shoots showed the fluorescence due to the *gfp* gene expression (data not shown).

This experiment was designed to provide proof of concept of successful handling of plastid transformation protocol in the laboratory using *gfp* sequences as reporter gene, *Prrn* promoter and *rps16 /psbA* terminator sequences (Sidorov *et al.,* 1999, Maliga, 2003) and 5 amino acid as enhancer sequence (Herz *et al.,* 2005). This chapter reports the successful generation of transplastomic GFP plant from three out of four plasmids used in the transformation experiments and none of the transplastomic shoots were obtained from pNAV206 plasmids (Table 4.1).

In conclusion, *gfp* has been commonly used as the reporter gene for nuclear, mitochondrial and plastid transformation experiments in variety of plant species (Pang *et al.,* 1996, Chiu *et al.,* 1996, Ghorbel *et al.,* 1999, Köhler *et al.,* 1997, Vain *et al.,* 1998). The overall plastid transformation frequency in this experiment was 1 transformed shoot obtained from 4 bombardments. For pNAV205 transformation frequency was 65%, which was closer to half the frequency of transplastomic shoot per bombardment as reported in tobacco (Svab and Maliga, 1993). This chapter proved that methods used for the plastid expression cassettes was successful and the plastid expression cassettes containing *cbhI* should produce stable transplastidic plants having similar level of CBHI as that of GFP.

Chapter 5: Heterologous expression of the cellobiohydrolase I gene in *Escherichia coli*

5.1 Introduction

The gram-negative bacterium *E. coli* is routinely used as a host organism for cloning of foreign DNA in plasmid vectors (Lodish *et al.,* 2000). A typical cloning vector contains an origin of replication, a selectable marker gene, and regions encoding multiple cloning sites and perhaps a scorable marker gene with promoter and terminator sequences. Recombinant protein production from *E. coli* have applications in therapeutics, diagnostics and industrial purposes (Eiteman and Altman, 2006).

Plasmid copy number in *E. coli* can vary from 5-500 copies per cell (Choi *et al.,* 1999, Ramos *et al.,* 2004). The plasmid copy number is regulated by factors such as metabolic load and recombinant protein produced in the host organism (Tegel, 2013). The expression of the recombinant protein in *E. coli* depends on several factors such as; promoter choice, DNA transcription level, codon usage, RNA translation, protein folding and stability (Sørensen and Mortensen, 2005). However, the metabolic load is dependent upon insert size within the plasmid, growth temperature, recombinant protein expression level, cell growth rate, nutrients, culture conditions and the toxicity of the expressed proteins that collectively affect the growth rates of the plasmid bearing cells (Palomares *et al.,* 2004). Nevertheless, in *E. coli* the promoter plays an important role in gene expression. Constitutive promoters are used when recombinant proteins have no effect on the growth rate of the host. *Prrn* is an example of a constitutive type promoter. *Prrn* is a σ70-type

promoter for the *16S rRNA*, the gene that encodes part of the chloroplast ribosomes (Sriraman *et al.,* 1998, Maliga, 2002, Vera and Sugiura, 1995). *Prrn* promoter consists of sequences in between the -35 and -10 region upstream from the transcription start site and resembles sequences of *E. coli* promoters (Hawley and McClure, 1983). In this project the chloroplast expression cassettes were designed using *Prrn* promoter that fuctions in both the host *E. coli* and in the tobacco plastids (Kuroda and Maliga, 2001) to produce increased level of expressed CBHI. The *Prrn* promoter is commonly used for plastid transformation experiments to ensure high levels of mRNA (Zoubenko *et al.,* 1994, Ruf *et al.,* 2001, Okumura *et al.,* 2006, Ruhlman *et al.,* 2010) and the 3'-region of the *rps16* ribosomal protein gene (*Trps16*) (Zoubenko *et al.,* 1994).

The expression cassettes in this study contain coding regions of either the full length or the catalytic domain of *cbhI* cloned in *E. coli* DH5α using the pUC18 vector backbone (Chapter 2). Since the expression cassettes of both *cbhI* and *gfp* vector are same that contains common *Prrn* promoter, it was predicted that CBHI get expressed in *E. coli* as well as in the chloroplast (Chapter 4).

The overall aim of this study was to examine recombinant expression of cellulases in chloroplasts, and in order to undertake these studies large amount of purified and intact *cbhI* vectors were needed. However, the host *E. coli* strains (DH5α and CopyCutter 1400) containing *cbhI* vector consists of small colonies on agar plates with low plasmid yield and rearrangements of plasmid DNA when cultures grown under standard conditions. Therefore, this chapter is about optimising the growth condition of the *E. coli* host harbouring the *cbhI* plasmids to obtain intact plasmids. Since temperature plays an important role for the stability and integrity of the plasmids that contain the genes for toxic proteins within *E. coli* (Baneyx, 1999),

the experiment was designed to optimise yield and integrity of plasmids at three different temperatures (37^{o}C, 30^{o}C and 25^{o}C).

5.2 Materials and methods

For growth curve analysis, 1 mL of test culture grown at room temperature (Table 5.1) was added in 50 mL Lb broth containing 300 mg/L of penicillin in 250 mL flask. The triplicates test cultures were set up using above cultures inoculum and each set were incubated in three different shaking incubators preset individually at 37^{o}C, 30^{o}C and 25^{o}C and cultures were allowed to grow over 24 h. Details of plasmids (pNAV223, pNAV225, pNAV227, and pNAV229) were used for the growth curve analysis are listed (Table 5.1). All the cultures containing *cbhI* and *gfp* expression cassettes were subcultured from strain storage ampules (stored in -80^{o}C freezer, maintained by Kim Stevenson in the plant laboratory RMIT University).

Table 5.1: The *cbhI* plastid expression cassettes

Plasmid	Expression cassette
pNAV222	*Prrn-cbhI* FL-*rps16*
pNAV223	*Prrn-cbhI* FL-*psbA*
pNAV224	*Prrn* (k)-*cbhI FL-rps16*
pNAV225	*Prrn* (k)- *cbhI* FL-*psbA*
pNAV226	*Prrn* (k)-*cbhI* cat-*rps16*
pNAV227	*Prrn*- cat-*cbhI –psbA*
pNAV228	*Prrn* (k)-cat -*cbhI*-rps16
pNAV229	*Prrn* (k)- cat- *cbhI –psbA*
pNAV204	*Prrn* (k)-*gfp*-psbA

The culture density was checked by reading the OD_{600} value at 4 h interval for about 24 h time period.

5.2.1 DNA quality determination

DNA was isolated from all the cultures using Promega DNA isolation mini kit. The integrity of both uncut and cut plasmids DNA with restriction enzyme EcoRI was checked using gel electrophoresis.

5.2.2 TSP extraction from *E. coli*

The cell pellets obtained from 10 mL cultures (grown at 37^{o}C and 24 h time period) spun at 5000 xg for 5 min. The cell pellets were resuspended in 500 µL of 50 mM sodium acetate buffer pH 5. Cells were disrupted by four cycles of snap thawing in

liquid nitrogen and then incubating at 42^{o}C in a water bath. The mixtures were spun, and the TSP was concentrated with Viva spin 10,000 MWCO PES column and concentrations of TSP recovered were about 1 µg/µL. Ten µg of the sample was mixed with protein loading dye containing DTT heated to 95^{o}C for 5 min. For the insoluble protein fraction, the residual pellet was resuspended in 100 µL of 50 mM sodium acetate pH 5. The genomic DNA was cleared by adding DNAase into the mixture followed by incubating at a room temperature for 30 min, then the sample buffer /DTT mix was added. The tubes were heated to 95^{o}C before loading protein extracts to SDS-PAGE gel.

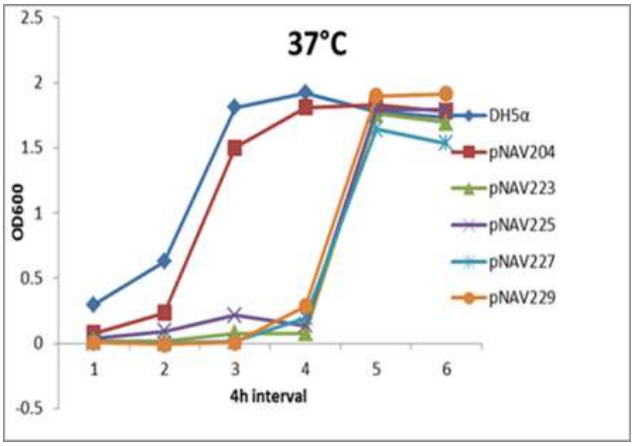
5.3 Results

5.3.1 Growth curve

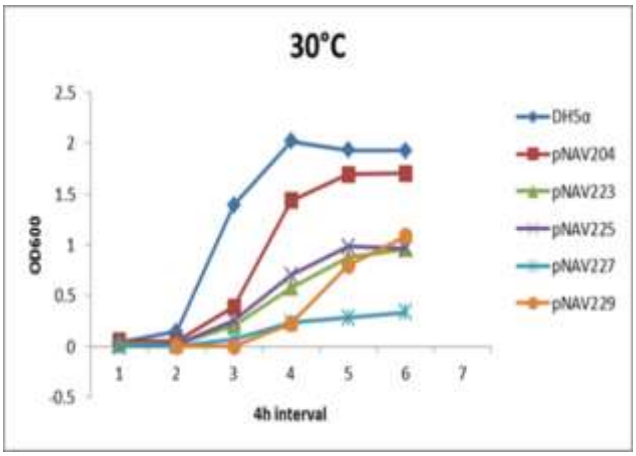
The growth curves of *E. coli* containing different plasmids at three temperatures are shown in Figure 5.1. At 37^{o}C DH5α (empty vector) and the DH5α containing pNAV204, reached exponential growth after 2 h and cultures harbouring pNAV223, pNAV225, pNAV227, and pNAV229 plasmids did not reach exponential phase until after 16 h. At 30^{o}C, all cultures containing *cbhI* plasmids had exponential phase between 12 h to 20 h, while the control GFP and DH5α strains had exponential phase from 10-16 h interval (Figure 5.1b). At 25^{o}C DH5α strain reached exponential phase by 5 h and strains containing *gfp* and *cbhI* plasmids had exponential phase at 12 h (Figure 5.1c). The slowest growing culture was pNAV227 (Figure 5.1c).

5.3.2 Gel analysis of the plasmid bands

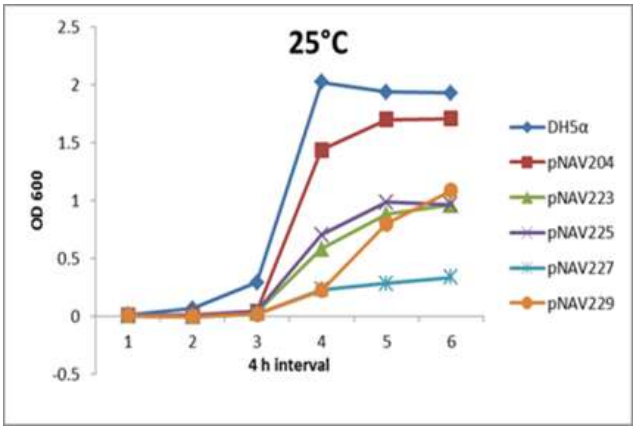
Plasmid DNA extract isolated from each of the cultures at 24 h was EcoRI digested to determine their respective integrity and purity. The rearrangement of the *cbhI* plasmids isolated from *E. coli* grown at 37^{o}C and 30^{o}C demonstrated by characteristic multiple bands in cultures containing *cbhI* expression cassettes (Figure 5.2), while the pNAV204 plasmids were intact in all the temperature ranges (Figure 5.2).



a.



b.



c.

Figure 5.1: Bacterial growth curves for *E. coli* strains grown at 37^{o}C, 30^{o}C and 25^{o}C, containing *Prrn-gfp* and *Prrn-cbhI* constructs, LB medium with 250 mg/L penicillin. The horizontal axis shows time in 4 h intervals and the vertical axis shows the optical density (OD at 600 nm).

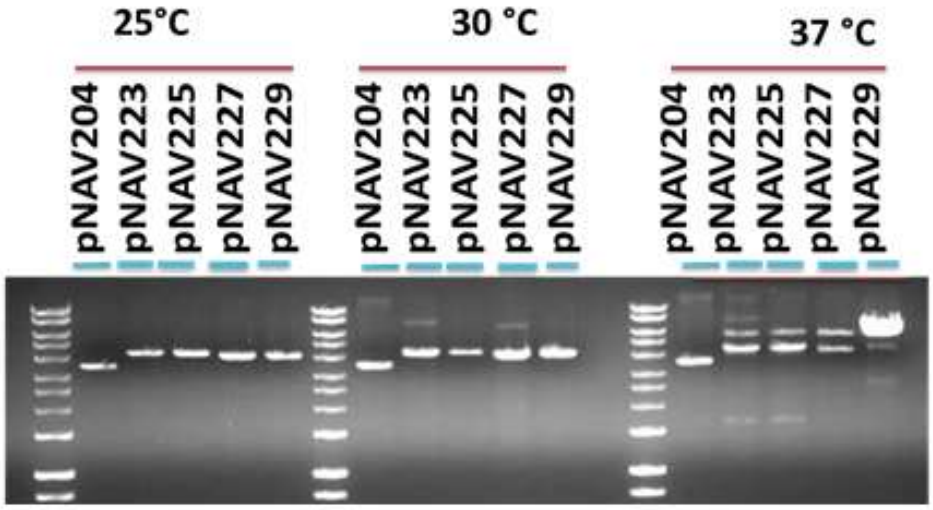
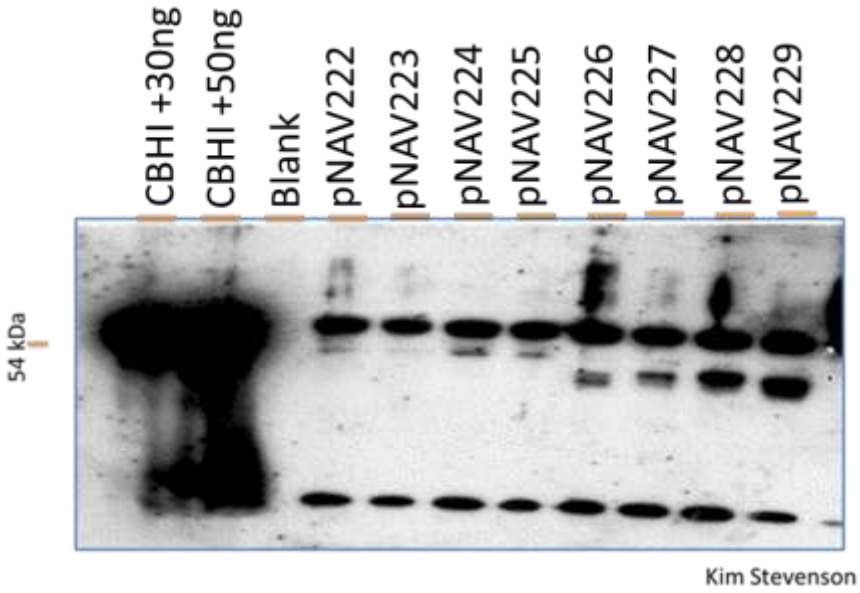


Figure 5.2: EcoRI digested plasmids from *E. coli* DH5α including pNAV204 (*Prrn-gfp*), and various *Prrn-cbhI* constructs such as; pNAV223, pNAV225, pNAV227 and pNAV229 grown at 30˚C, 37˚C and 25˚C.

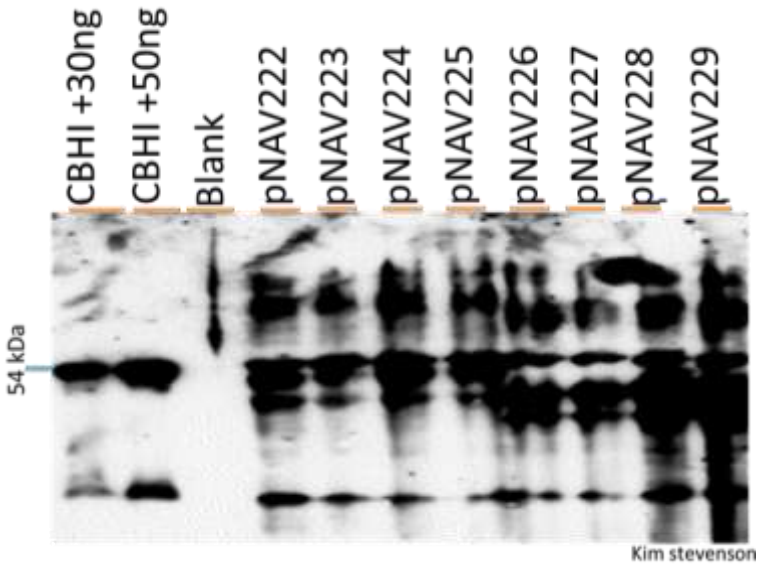
5.3.3 Western blot

CBHI expressions of TSP extracts of *E. coli* transformed with *Prrn-cbhI* plasmids were confirmed by western blot. Expression of both the full length and catalytic domain of CBHI are shown in Figure 5.3. Cells containing plasmids encoding the full length of CBHI (pNAV222, pNAV223, pNAV224 and pNAV225) or catalytic domain of CBHI (pNAV226, pNAV227, pNAV228 and pNAV229) produced cross reacting band of 56 kDa and 46 kDa respectively.

There was no MUC activity in both the TSP and insoluble extracts of *E. coli* expressed CBHI (data not shown).



a. Western blot of *E. coli* CBHI with TSP



b. Western blot of *E. coli* containing CBHI plasmids total insoluble protein (TIP)

Figure 5.3 Western Blot of *E. coli* lysates containing *Prrn-chbI* constructs (full-length: pNAV222, pNAV223, pNAV224 and pNAV225 and catalytic domain: pNAV226, pNAV227, pNAV228 and pNAV229) and pNAV204 (*Prrn-gfp*), a. Total Soluble Protein (TSP) extracts and b. insoluble extract.

5.4 Discussion

The abnormal growth of *E. coli* cells containing *cbhI* plasmids might be due to double membrane structure of *E. coli* cells which leads to the accumulation of expressed CBHI causing a metabolic burden. The reduction of copy number of plasmids were reported previously (Jones and Keasling, 1998, Jones *et al.,* 2000). The plasmid reorganization might be due to the response of the metabolic burden caused by the recombinant protein produced within the *E. coli* host cells (Silva *et al.,* 2012). *E. coli* expressed CBHI might have formed the inclusion bodies thereby affecting the endogenous protein stability and integrity of the plasmids. The low copy number of the plasmids was associated with toxicity of the recombinant protein (Sørensen and Mortensen, 2005) resulting in the formation of the inclusion bodies and other aggregates formed during protein folding in *E. coli* (Liu *et al.,* 2013). The expressed CBHI leading to the formation of inclusion bodies have been reported in *E. coli* (Fahnert *et al.,* 2004, Gonzalez-Montalban *et al.,* 2005, Rosano and Ceccarelli, 2009). The results in this chapter agree with the argument leading to the forming inclusion bodies as CBHI was detected both in the TSP and insoluble fractions (Figure 5.3). However, further examination for inclusion bodies formations were not carried out as this step was beyond the scope of this project.

At 25^{o}C all the plasmids (*Prrn-cbhI* and a *Prrn-gfp*) are intact and had almost same yield. Plasmid rearrangements were observed in cultures (pNAV223, pNAV225, pNAV227 and pNAV229 plasmids) grown at 30^{o}C and 37 ^{o}C (Figure 5.2) and these plamids could not be used for plastid transformation experiments. These problems were corrected by growing *E. coli* cultures at 25^{o}C over 24 h period and intact plasmids thus obtained were used for transformation experiments.

Chapter 6: Expression of *Trichoderma reesei* cellobiohydrolase I (cel7A) in transplastidic tobacco via particle bombardment

6.1 Introduction

Tobacco plastids have high ploidy genome with their own transcription-translation machinery and the compartmentalisation of proteins allow high levels of foreign protein expression up to 70% total soluble protein (Viitanen *et al.,* 2004, Kumar *et al.,* 2004, Oey *et al.,* 2009). The advantage of using plastid transformation over nuclear expression are several which includes high levels of recombinant protein, transgenic containment via maternal inheritance, and multigene expression in single transformation event (De Cosa *et al.,* 2001; Verma and Daniell, 2007). Furthermore, plastids have the ability to form disulfide bonds which helps in folding of complex proteins in the stroma and other post-translational modification required for the protein maturation (Cardi et al., 2010; Dunne *et al.,* 2014).

The *E. coli aadA* is the most commonly used selectable marker gene for the plastid transformation due to its high specificity and limited site-effects in selecting the transgenic plants from the transformed cells (Dunne *et al.,* 2014). The *aadA* through adenylation prevent both antibiotics (spectinomycin and streptomycin) binding to plastid ribosomes, there by conferring resistant to the transformed cells.

There are multisteps processes involved to achieve the plastid transformation. First step is to develop plastid transformation vectors that flank the foreign genes and insert them into the precise location into the plastome via homologous recombination event. Biolistic method of delivery of the vector is the method of choice over polyethylene glycol (PEG)-mediated for the stable transformation of the tobacco

leaves. Generally there steps are involved during the plastid transformation of plants; the selection and regeneration of the bombarded shoots, homologous recombination event occurring at the selection phase and finally the generation of the transplastomic plants (Maliga and Bock, 2011).

Despite the expression of cellulases in plants via nuclear transformation, to date there are only a few reports of expression of CBHI *from T. reesei* in plants (Dai *et al.,* 1999a, Hood *et al.,* 2007, Harrison *et al.,* 2011). None of these reports were about expression from the plastid genome. Given the reported advantages of plastid based expression in plants, it was decided to challenge *cbhI* expression in the chloroplasts of the model species tobacco.

The differences in the contructs was in the 3'untranslated region and the presence or absence of a synthetic 5 amino acid translational enhancer sequence (k) in the upstream region of the constructs (Olins and Rangwala, 1989, Maliga and Bock, 2011).

The main aim of this chapter was to generate transplastomic plants each expressing one of four variants of an expression cassette containing a codon optimised with tobacco plastids version of the full length of *cbhI* coding region (Table 6.1).

6.2 Materials and methods

The materials and methods for the plastid transformation such as: maintenance of plant materials, plant transformation protocol, particle bombardment, regeneration of the spectinomycin resistant shoots, molecular analysis steps such as PCR, RT-PCR,

Southern blot and western blot analysis, chloroplast isolation, coomassie gel staining, transmission electron microscopy (TEM), have been described in Chapter 2. Several batches of *E. coli* cultures were set up to isolate *cbhI* plasmids that was combined to obtain desired amount and concentration and 50-70 bombardments were carried out using the prepared plasmids. Three sets of primers (set A, set B and set C) were designed for PCR analysis. The set A is for the *cbhI* region with expected product size of 1.3kb, *set* B spans from *aadA* to *16S rRNA* regions of the plastid genome with product size of 2.46 kb and primer set C spans from the *aadA* to *cbhI to rps12/7* region with an expected product size of 2.86 kb.

6.3 Results

6.3.1 Expression constructs

The expression vectors used for bomabarment experiments are listed in Table 6.1. The full-length codon optimised version of *cbhI* was designed to target the chloroplast *rps12/7* and *16S rRNA* intergenic region by homologous recombination.

Table 6.1: Selection of plastid transformants

Plasmid Vector	Expression cassette	Total shots	Spectinomycin resistant	Transformants
pNAV230	*Prrn-cbhI-rps12*	50	14	1
pNAV231	*Prrn-cbhI-psbA*	50	7	0
pNAV232	*Prrn(K)-cbhI-rps12*	50	6	1
pNAV233	*Prrn(K)-cbhI-psbA*	50	6	1

6.3.2. Phenotypes of *Prrn-cbhI* transplastomic plants

There were only 3 transplastomic plants one each from pNAV230, pNAV232 and pNAV233 lines. Transplastomic lines had altered phenotypes compared with the non-transformed plants. Pale leaves with reduced chlorophyll content, accompanied with slower growth and reduced size and thickness of the leaves in the three CBHI transplastomic lines. There was abnormal growth in the pNAV232 plastid transformed plants which do not develop roots, leaves and stem (Figure 6.1).

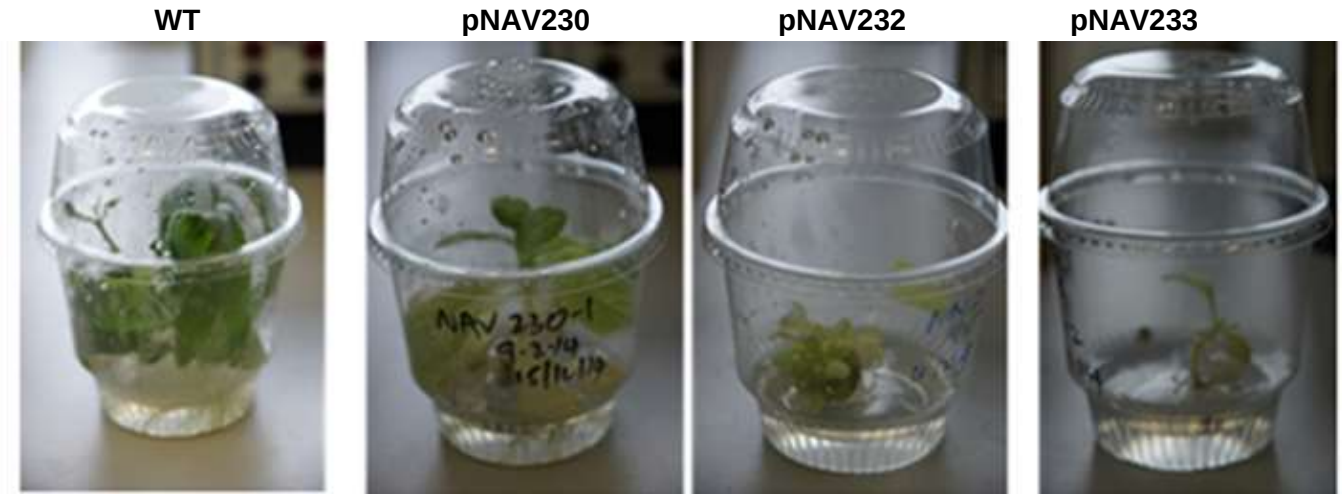


Figure 6.1 Phenotype of non-transformed (WT) tobacco and plastid transformed *Prrn-cbhI* lines pNAV230, pNAV232 and pNAV233.

6.3.3 PCR analysis

Putative transgenic shoots were screened by PCR (Figure 6.4) using gene specific primers (Table 2.2). Similarly, there were 14 spectinomycin resistant shoots from pNAV230, 6 shoots from pNAV232 and 7 from pNAV231 but only three transplastomic plants were confirmed, one each from pNAV230, pNAV232 and pNAV233 (Table 6.1). The transformation frequency with the *Prrn-cbhI* constructs in tobacco was extremely low (i.e total of 3 transgenics from 200 shots) compared to 13

transplastidic lines obtained from pNAV205 (*Prrn-gfp*) out of 20 shots (Table 4.1 Chapter 4). Unfortunately, no transplastomic line was recovered from pNAV231 vector from 50 bombardments.

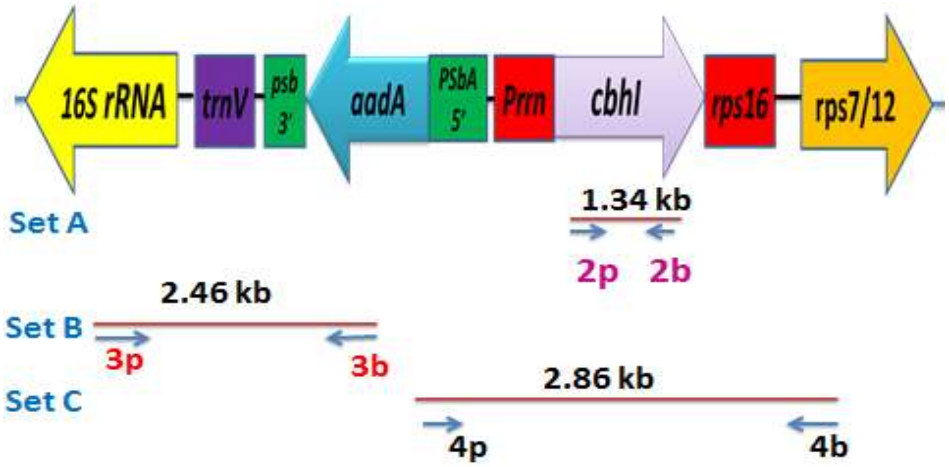
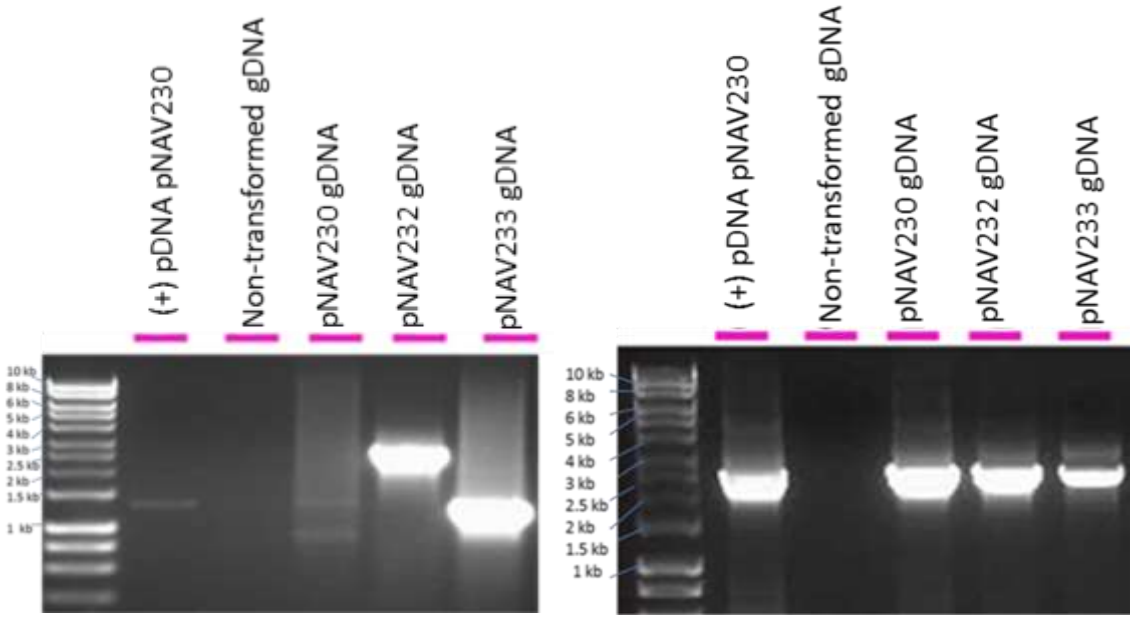


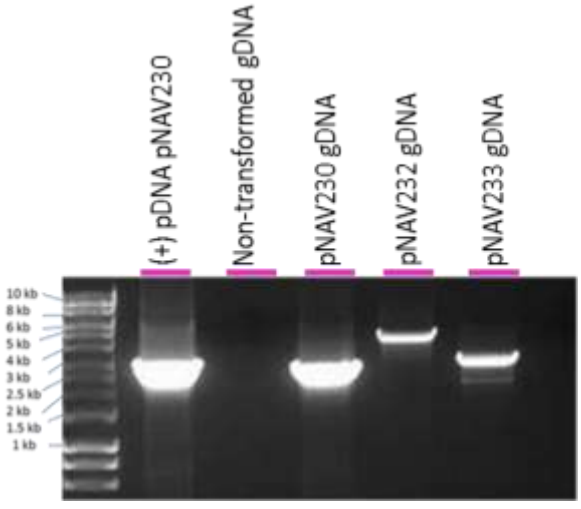
Figure 6.2 Map of cbhI expression cassette showing the point of integration in the tobacco chloroplast genome. Primer pairs used for confirming putative transplastidic plants are; Set A primers are for amplification of *cbhI* with an expected product size of 1.34 kb. Set B primer spans from *aadA* to *16S rRNA* with expected product size of 2.46 kb and set C amplifies from *aadA* selectable marker from *cbhI* expression cassette to *rps12/7* with expected size 2.86 Kb.

The PCR product obtained from set A primers are at the predicted size of 1.3 kb for pNAV230 and pNAV233 transformed plant genomic DNA (Set A primer Figure 6.3), but the product size in pNAV232 is at 2.8 kb. With primer Set B the entire PCR products are at the expected size of 2.46 kb (Set B primer Figure 6.3). The PCR product of set C primer pairs are at predicted size of 2.86 for pNAV230 and pNAV233 lines and for pNAV232 line the PCR product was at about 4.36 kb which was 1.5 kb larger than the other lines. There were two band observed in pNAV230 line with set A primer and in pNAV233 line with set C primer. From the PCR results it was concluded that insertion of an extra sequence of about 1.5 kb had occurred in

the *cbhI* sequences. The mechanism of the insertion is unknown, which is common in biolistic based transformation of both chloroplast and nuclear genomes.



Set A Primer Set B Primer



Set C Primer

Figure 6.3 PCR analyses of transplastomic *cbhI* (pNAV230, pNAV232, pNAV233-3) lines using above three sets of primer combinations. HyperLadderTM 1kb (Bioline). Positive control (+) pDNA pNAV230 (plasmid DNA from pNAV230).

6.3.4 Southern blot analysis

Southern blot analysis confirms the site-specific integration of expression cassettes and determines the homoplasticity of transplastomic CBHI plants. For Southern blot analysis genomic DNA was isolated using the Trizol method (Sigma-Aldrich, USA), digested with *Bgl* II and DNA hybridised with radiolabelled *cbhI* and *16S rDNA* probes.

The Southern blot hybridization confirmed the stable and site-specific integration of expression cassettes containing *cbhI* sequences and the selectable *aadA* into the tobacco plastid genome in all three transplastidics, which was consistent with the PCR results (Figure 6.3). Hybridisation with the flanking region (*16S RNA* probe) has confirmed site-specific integration of the expression cassettes into the intergenic region between trnV and rps7/12 genes (Figure 6.4 and 6.5b). Presence of low molecular weight band corresponding to the the non-transformed plant DNA band is a clear indication for the heterotransplastomic nature of their plastome (Figure 6.5b). The sharper and more denser band in the genomic DNA from pNAV230 transplastomic plant suggests that efficient transcription of transgenes occurred in the former plant than the pNAV233 transplastomic plant. The presence of high molecular weight band in pNAV232 transplastomic plant represented that rearrangement of transcripts occurred during transgene integration into the host genome of the plant (Figure 6.5 b). For example, using both the probes (*cbhI* and *16S rRNA*), it was confirmed that line pNAV232 had band at 8.9 kb and remaining two lines (pNAV230 and pNAV233) had bands at expected size 7.4 kb. This data corresponds to *cbhI* and *16S rRNA* probe that the PCR result for pNAV232 was at

1.5 kb larger than pNAV230 and pNAV233 plants. The hybridisation band for positive control was at 9.4 kb, this was from the orginal size of the *Bgl* II linearised pNAV230 plasmid DNA.

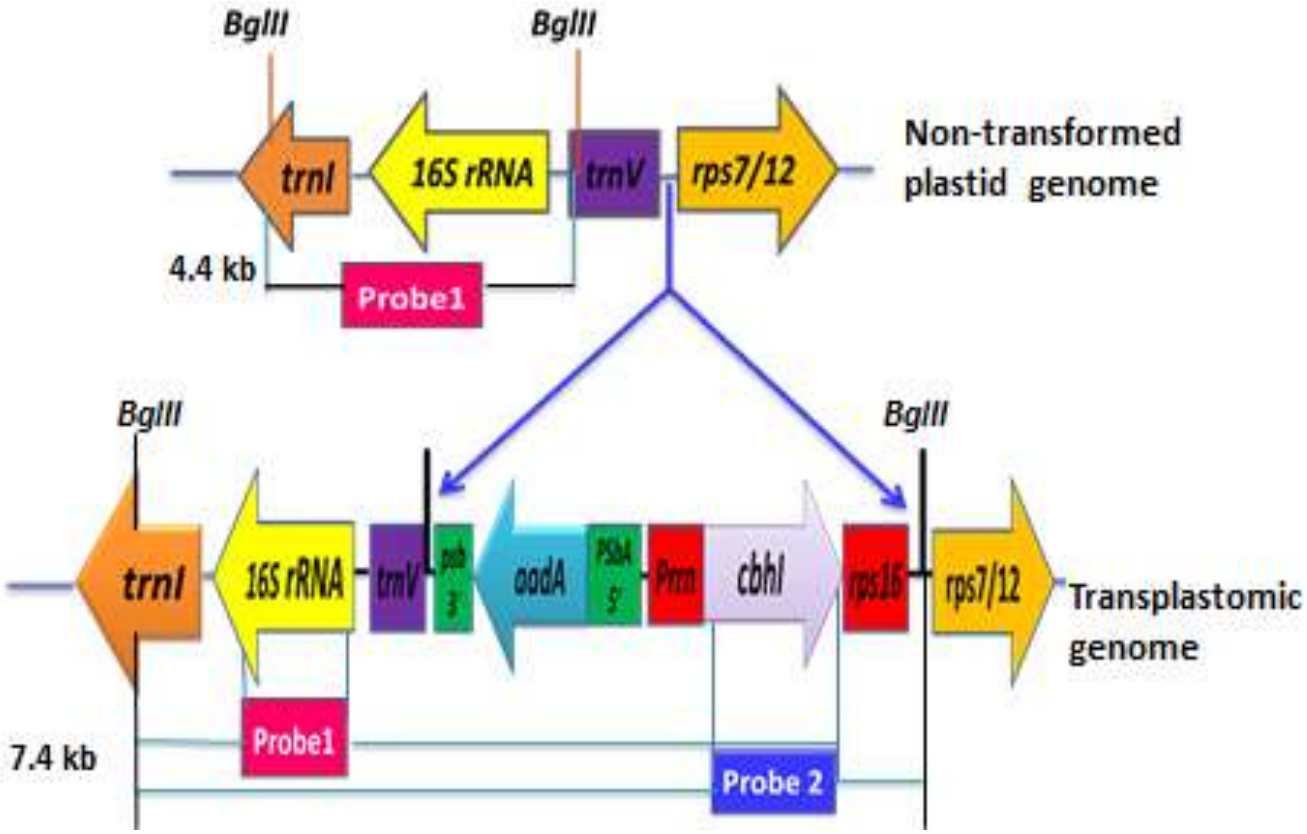
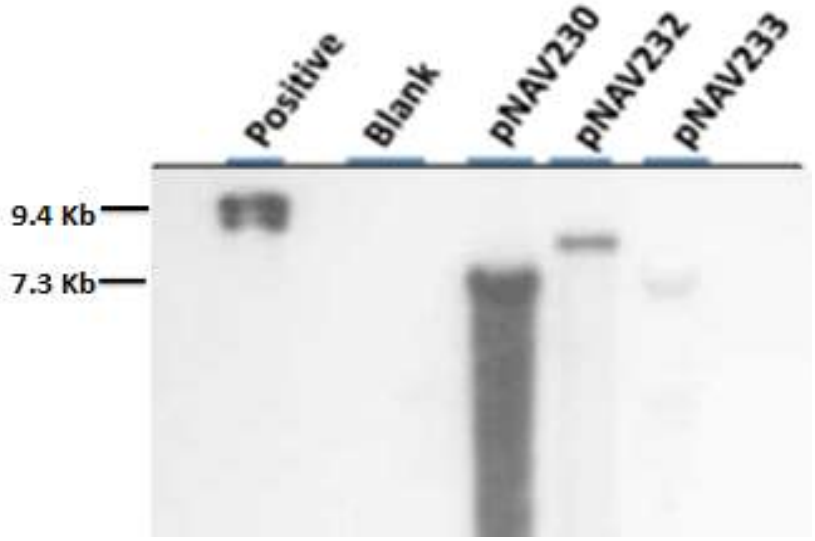
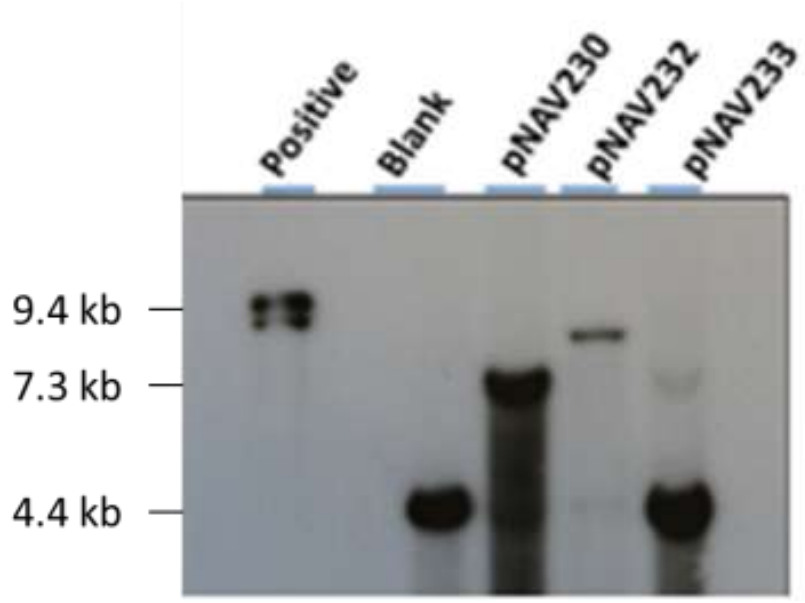


Figure 6.4 Map of codon-optimised *cbhI* into tobacco chloroplast: Physical map of intergenic region of wt plasmid DNA (pt-DNA) (upper) and the transgenic plastid genome of transplastidic *cbhI* plants (lower). Relevant *Bgl* II *restriction* sites used for Southern blot analysis are shown. The sizes of the expected restriction fragments are indicate



A. *cbhI* probe



B. *16S RNA* probe

Figure 6.5 Southern blot analysis of Bgl II digested genomic DNA from *cbhI* transformants.

6.3.5 Coomassie Brilliant Blue stained protein gel

Since the transplastomic leaves phenotype were pale (Figure 6.1), it was decided to determine the rubisco content in the TSP extracts of the transplastomic lines (pNAV230, pNAV232 and pNAV233). The crude extracts of pNAV230 plant run in

the protein gel found that rubisco levels were equal to non-transformed plant (Figure 6.6), however, rubisco level was reduced in pNAV232 and pNAV233 plants.

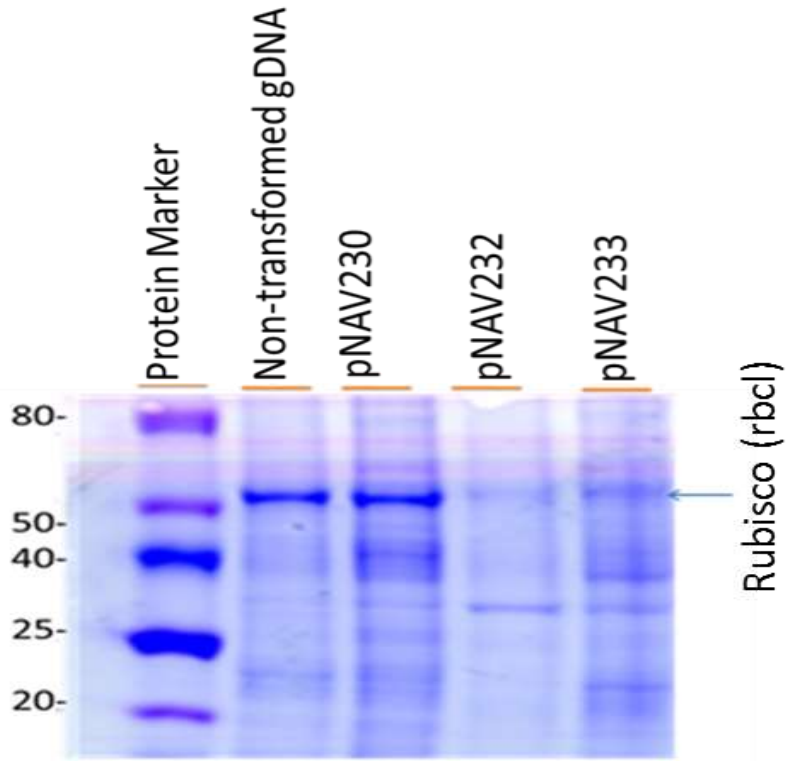
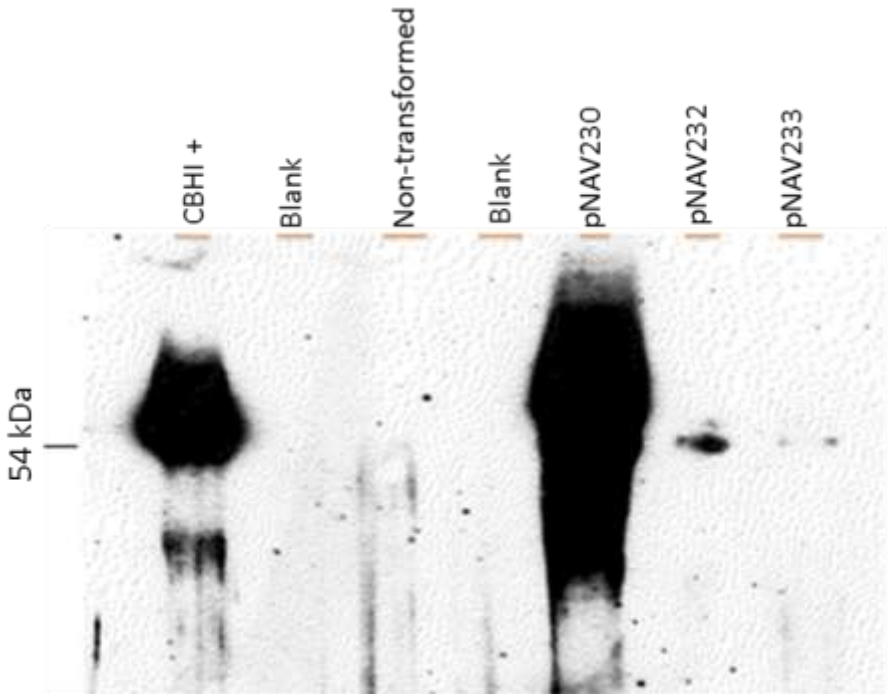


Figure 6.6 Coomassie Brilliant Blue strained protein gel for estimation of rubisco content in non-transformed and transplastomic CBHI lines.

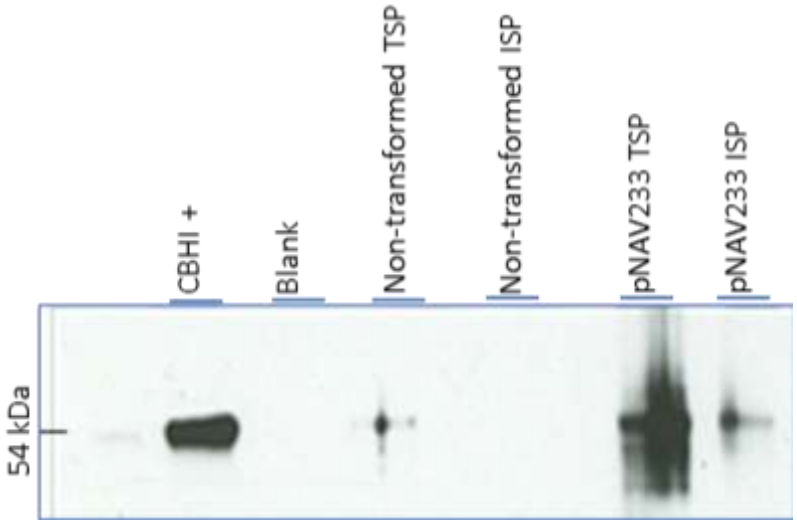
6.3.6 CBHI levels of transplastidic shoots

For the estimation of expressed CBHI in transplastomic plant extracts equal amount of TSP was loaded to the known amount of purified CBHI as standard. The western blot result in Figure 6.7 showed that expression level of recombinant CBHI was between 0.5% in pNAV230 and 0.02% for pNAV233 of total leaf protein (TLP). No CBHI band was detected for pNAV232 plants (Figure 6.7B). The amount CBHI protein produced can be compared to the Southern blot result that showed that more transgene in pNAV230 plant compared to that of pNAV232 and pNAV233

transfomed plants. The second western blot was run for pNAV233 line using chloroplast enriched protein as it was not possible to detect CBHI in first western blot results (Figure 6.7A). The chloroplast enriched TSP of pNAV233 line showed cross reacting band with CBHI antibody (Figure 6.7B). However, it was not possible to isolate the chloroplast from pNAV232 line due to limited leaf biomass. The cross-reacting bands for CBHI obtained were at expected molecular weight of 54 kDa in both pNAV230 and pNAV233 transplastomic lines.



A.



B

Figure 6.7 Immunodetection of CBHI. (A): Purified CBHI (50 ng) from *T. reesei,* blank (extraction buffer), control (20 µg of TSP extracts of non-transformed tobacco leaves extracts), TSP leaf extracts (20 µg each) from transformants: pNAV230, pNAV232, pNAV233 plants. (B): Purified CBHI (25 ng) from *T. reesei*, blank, non-transformed TSP, non-transformed insoluble protein (ISP), pNAV233 TSP and pNAV233 ISP.

6.3.7. RNA transcript levels for *Prrn-cbhI in* transplastidic shoots.

RT-PCR analysis was conducted to test the transcripts level of plastid genes and *cbhI* transcripts of cDNA obtained from total RNA extracts (Figure 6.8). Actin gene primer pairs used for determining the mRNA load in the template and the products showed a uniform band reflecting similar levels of actin mRNA in the test samples. The RT-PCR results using *cbhI* primers produced bands in pNAV230 and pNAV233. None of the *cbhI* transcripts were detected from pNAV232 lines. Reduction in mRNA level in pNAV232 and pNAV233 transplastidic lines were observed when RT-PCR data compared with that of non-transformed and pNAV230 transplastidic plant. Corresponding to this, line pNAV232 had the lowest signal of mRNA in three plastid genes tested in this experiment.

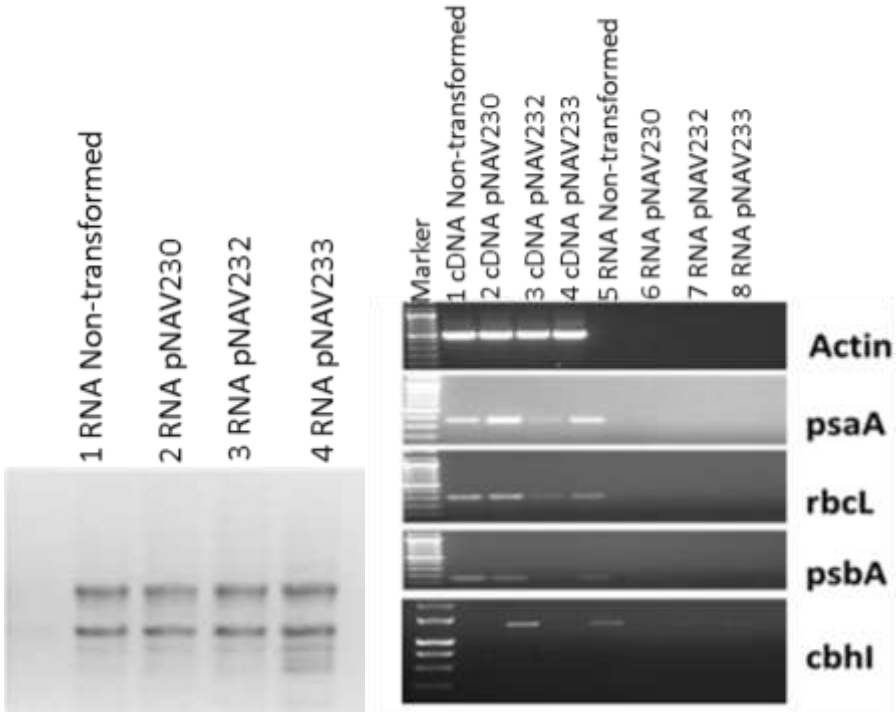


Figure 6.8 RT-PCR analyses of the non-transformed and transplastomic CBHI lines with house-keeping gene primers and gene specific primer pairs. A. Total RNA samples stained with ethidium bromide and B. RT-PCR product with primers pairs (Actin, *psaA, rbcL, psbaA* and *cbhI*).

6.3.8 Chloroplast ultrastructure

The differences in ultrastructure of chloroplast in the mesophyll cells of non-transformed tobacco and transplastomic plants (both GFP and CBHI) were analyzed by electron microscopy. It was shown that ultrastrucural organisation of chloroplast of transplastomic CBHI plants had distinct difference from the chloroplast of transplastomic GFP to that of non-transformed plants by numbers, arrangement of thylakoids, number of sizes of starch grains and plastoglobules. For example, in pNAV233 transplastomic plant chloroplast had disorganised thylakoid membrane (Figure 6.9). The chloroplasts of pNAV233 had condensed thylakoid membrane while it was separated in pNAV230 resulting into thin granum stacks. The thylakoid membrane was elongated in pNAV232 chloroplast and frequent deposition of oil bodies called plastoglobulins was observed when compared to the non-transformed and pNAV205 transplastidic plants.

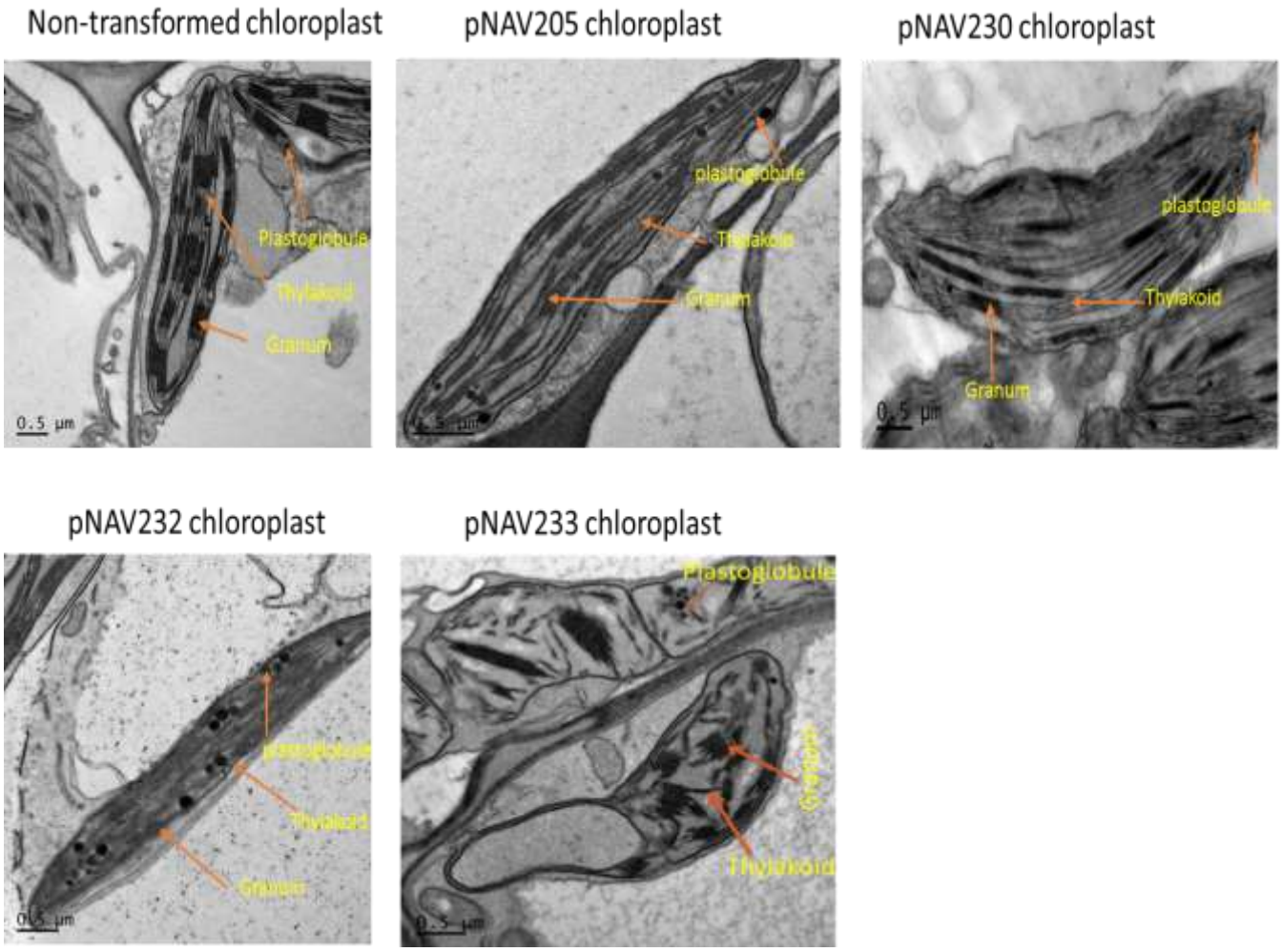


Figure 6.9 TEM images displaying chloroplast ultrastructure of non-transformed plant versus GFP positive and CBHI transplastomic plants.

6.3.9 Enzymatic activity

There was no activity of transplatomic CBHI line with MUC substrate (data not shown).

6.4 Discussion

There are substantial numbers of reports of *in planta* expression of different cellulases targetted to different cellular compartments such as vacuoles, endoplasmic reticulum, apoplast, mitochondria and chloroplasts (Hood and Requesens 2012, Garvey et al. 2013). There were varying degrees of expression of CBHI in transgenic plants such as tobacco (0.02% of TSP) (Ziegelhoffer *et al.,* 1999, Dai *et al.,* 1999b), corn (Hood *et al.,* 2007, Park et al. 2011) and sugarcane (Harrison *et al.,* 2011) with accumulation of CBHI up to 1% of TSP in leaves or up to 16% in corn kernels but the plastid expression of CBHI has not been reported previously.

This chapter reports the successful insertion of *T. reesei cbhI* in the transcriptionally active spacer region of tobacco plastid genome in between *trnV* and *rps12/7* for the first time attained about 0.5% (Figure 6.7 A) of CBHI expression in plastids. However, this chapter prompted that further examination is required to explain why *cbhI* results into low transformation frequencies, change the ultrastructure of the chloroplast, production and interaction of CBHI with native host plant protein and factors affecting the drastic change in the plant phenotype of *cbhI* lines. Similar report of this type of lethal effect of transplastidic *cbhI* lines with characteristic chlorotic leaves with disrupted chloroplast ultrastructure and expressing cell wall degrading enzymes in tobacco had been reported previously (Agrawal *et al.,* 2011, Petersen and Bock, 2011). Furthermore, there was reduced transcript of housekeeping genes in pNAV232 and pNAV233 than pNAV230 and non-transformed lines, and the basis of this is not fully understood.

As stated in the beginning of this project that one of the major challenge of producing cellulosic ethanol depends upon the availability of large amount of CBHs

and its production yield via heterologous system was inadequate (Seiboth *et al.,* 2011). The main strategy developed at the commencement of this project was to produce large amount of functionally active CBHI via transplastomic approach in tobacco which would be radially available with other cellulases required during the digestion of the cellulose. The results of transplastomic CBHI plants had pigment-deficient mutant phenotypes that had detrimental effect on subsistence or growth and development of plants even at minimal expression of CBHI, this result contrasted with detrimental effect caused by over expression of the cellulolytic enzymes (Petersen and Bock, 2011). However, in this project generation of transplastomic tobacco plants expressing CBHI had been accomplished though it could not produce functionally active CBHI to digest cellobiose unit. This chapter concludes that possibility to engineer in the plastid genome of higher plants with *T. reesei* CBHI would have similar impact of producing functionally inactive enzymes with detrimental effect on the ultrastructure of the chloroplast which altered the gowth and development of transplastomic plants.

Chapter 7: Tobacco plastid transformation of a gene for a protozoan exocellulases from termite

7.1 Introduction

Insects have evolved to produce endogenous and symbiotic enzymes to efficiently digest lignocellulosic material as a source of metabolic glucose. The potential for cellulose degradation has been widely observed in insects, having been reported in eight orders, 20 families and 78 species (Panizzi and Parra, 2012). Termites belong to family Isoptera and have evolved to play an important role in the carbon cycle of ecosystems by utilising lignocellulosic substrate either by producing their own lignocellulases or by harbouring a cellulolytic intestinal micro-flora (like bacteria and archaea) and fauna (protozoans) (Martin, 1983, Tokuda *et al.,* 1997, Yamada *et al.,* 2005). The most primitive termite *Mastotermes darwiniensis* belongs to family Mastotermitidae harbours 4 flagellates including *Koruga bonita*, *Deltotrichonympha nana*, and *D. operculata* and the trichomonad *Mixotricha paradoxa* (Li *et al.,* 2003). There are about 2,600 species of termite and 281 genera (Kambhampati and Eggleton, 2000). Termites are member of taxonomic orders of such as; Isoptera (Martin and Martin, 1978), Thysanura (Treves and Martin, 1994), Coleoptera (Genta *et al.,* 2006), Orthopera (Cazemier *et al.,* 1997), Plecoptera, Trichoptera, Diptera and Hymenoptera (Watanabe and Tokuda, 2010) have the ability to digest lignocellulosic biomass and are often problematic pests that can cause considerable damage to human built structures and commodity products (Zhou *et al.,* 2008). For example, the termite *Coptotermes formosanus* is a pest species that feeds on wood and is cosmopolitian in distribution and harbour three symbiotic hypermastigotes (any member of the zooflagellates protozoan living in termite digestive tracts):

Spirotrichonympha leidyi, *Holomastigotoides mirabile* and *Pseudotrichonympha grassii.* (Yamin, 1979, Ohkuma *et al.,* 2000). There are two types of cellulase systems in termites, the first type belongs to cellulases produced by termite are endogenous cellulases of GH9 family (Watanabe and Tokuda, 2001) and second type of cellulases belongs to that produced from their symbiotic protozoa of the families GH5, 7 and 45 (Watanabe *et al.,* 1998, Ohtoko *et al.,* 2000, Davison and Blaxter, 2005). Termites can digest about 74-99% of the cellulose and about 65-87% of the hemicellulose components of the lignocellulose they injest (Ohkuma, 2003). Since termites are well-known cellulose decomposers, its potential to be used as a supplement in the enzymatic hydrolysis of cellulose was expected to be achieved through heterologous expression of the cellulases.

7.2 Challenges of heterologous expression of termite cellulase genes

There has been limited success in heterologous expression of cellulases from termite origin (Ni *et al.,* 2005). The cellulases from various termites such as (N*asutitermes takasagoensis* (Khademi *et al.,* 2002), *S. frugiperda* and *Coptotermues formosanus* were expressed in *E. coli*, (Marana *et al.,* 2004, Zhang *et al.,* 2009) and β-glucosidase from the termite *Nasutitermes takasagoens* is expressed in yeast (*Pichia pastoris*) (Uchima, 2012). Studies have shown that expression of β-glucosidase cDNA fr*om Neotermes koshunensis* in *E. coli* had three fold increase in the specific activity than the natively produced protein (Ni *et al.,* 2007). The transcript of RsSymEG (an endoglucanase of glycosyl hydrolase family (GHF 7) isolated from the symbiotic protist of the termite *Reticulitermes speratus*

when expressed in *A. oryzae* could efficiently hydrolyzes the β-1,4-cellulosic linkage of cellodextrins into cellobiose and glucose (Todaka *et al.,* 2010). The β-glucosidase from the termite (*Nasutitermes takasagoensis*) expressed in *Pichia pastoris* was found to be active at 60^{o}C (Uchima, 2012). However, detailed analysis of cellulases expression in plant host has not been performed.

The main aim of this chapter is to explore the possibility of generating functionally active exocellulase from a termite gut protozoan via transplastomic tobacco as an alternative to address the challenge of obtaining functionally active *T. reesei* CBHI (Chapter 6). The production of functionally active plant expressing CO14 would be an alternative to the complications in expression of *T. reesei* CBHI discussed in Chapter 6.

7.3 Materials and methods

Total RNA extracts from termite gut microflora was used to make cDNA library. Then pCH151 vector containing *co14* was kindly provided by former RMIT employee scientist Dr. Chung Hong Chen (former CSIRO scientist). The expression cassettes pMAT-T vector (*HindIII, NcoI* and *PstI* sites) contains constitutive promoter sequence *Prrn* and *rps16* terminator. Both pMAT-T vector and pCH151 constructs were assembled for *co14*. Both vector pMAT-T and pCH151 were restriction enzyme digested with PstI and NcoI and ligation of the two fragments were carried out with standard cloning protocol to obtain the pNAV308. Final plastid expression vector pNAV310 was constructed using pNAV208 and pNAV308 digested with HindIII and NotI ligation of the fragments (Figure 7.1).

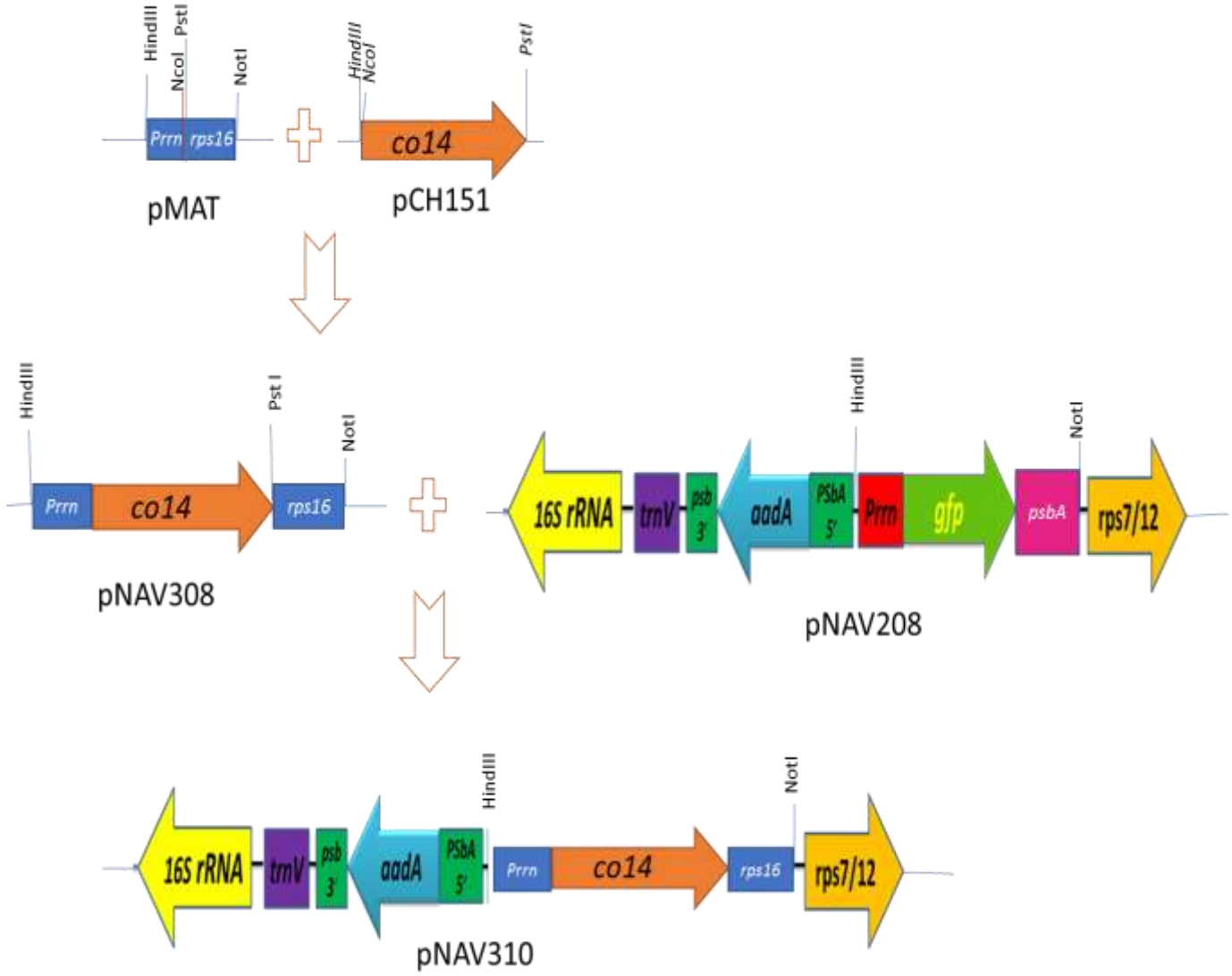


Figure 7.1 Construction of *co14* chloroplast homology vector. The *Prrn* promoter and *rps16* terminator are derived from pMAT vector. Both pMAT and pCH151 vectors digested with PstI and NcoI and digested fragments were ligated to obtain pNAV308 that contain expression cassettes *Prrn-co14-rps16.* Restriction enzymes digest of pNAV308 and pNAV208 at HindIII and NotI sites then ligated to obtain pNAV310. The resulting pNAV310 vector contains expression cassettes (*Prrn-co14-rps16*) and *aadA* selection marker and homologous recombination sequence (*16S rRNA* and *rps7/12*) at the ends.

7.4 Results

7.4.1 Regeneration of pNAV310 shoots after bombardment

Plastid transformation of tobacco leaves with pNAV310 vector was carried out by biolistic protocol (Khan and Maliga, 1999). All together there were six spectinomycin resistant shoots (pNAV310-1, pNAV310-2, pNAV310-3, pNAV310-4, pNAV310-5, and pNAV310-6) obtained from 20 bombardments. From the initial PCR analysis using gene specific primers pNAV310-1 and pNAV310-6 lines were positive, and the lines were maintained in MS medium with spectinomycin (500 mg/L) for further molecular analysis.

7.4.2 PCR analysis to confirm transgene integration

For PCR analysis, three primer sets were used. Three primer pairs Set A, Set B and Set C were used to amplify products of predicted sizes of 1.3, 2.46 and 2.86 kb respectively (Figure 7.2). Two putative transgenic shoots (pNAV310-1 and pNAV310-6) were analysed by PCR with 3 primer sets (Figure 7.2). The PCR showed that expected size product obtained from all the primer pairs as shown in the figure 7. 3.

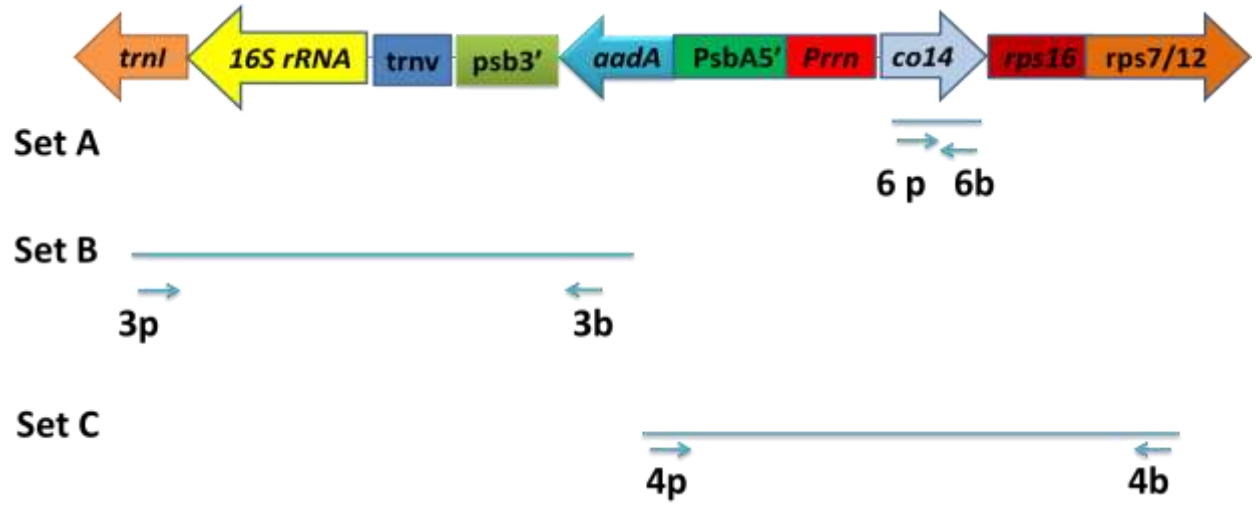


Figure 7.2 Map of the chloroplast expression vector shows the integration sites, 16SrRNA and rps7/12, *aadA* selectable marker genes, and *co14*.

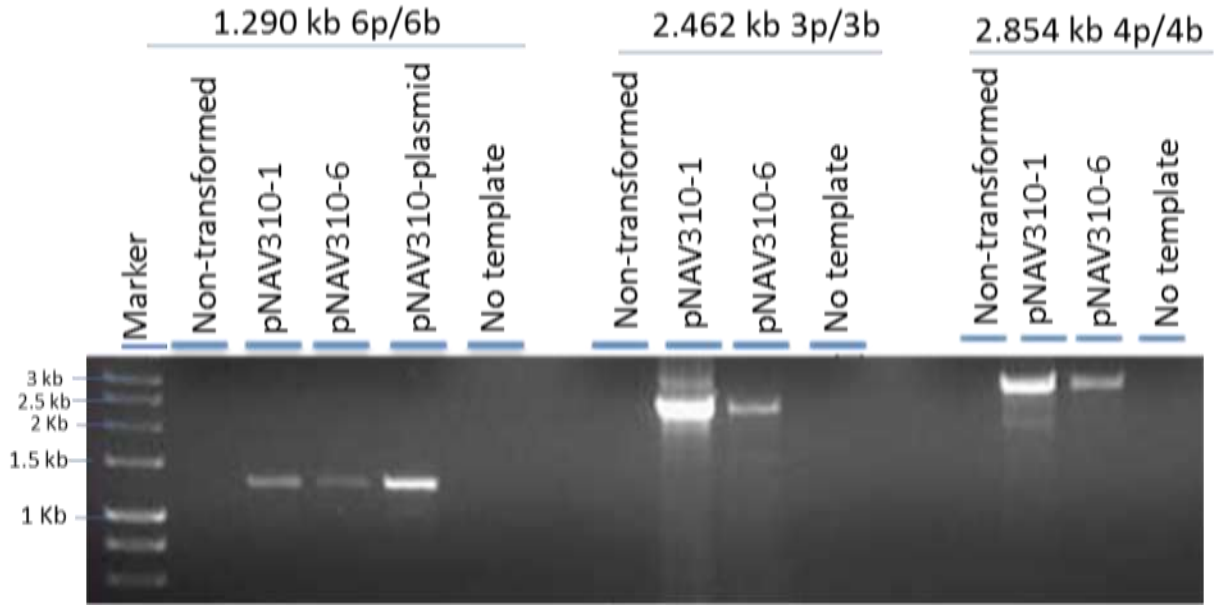


Figure 7.3 PCR screening of putative transformed shoots with 3P/3b; 6P/6b and 4P/4b primer pairs.

7.4.3 Southern blot analysis

Southern blot analysis confirmed the integration of *co14* and *aadA* into the tobacco plastid genome (Figure 7.4). Hybridization with the flanking region (*16S rRNA*) has

confirmed site-specific integration of expression cassettes into the intergenic region between *16S rRNA* and *rps7/12* genes (Figure 7.2). In pNAV310-6 plant there was absence of band corresponding to the low molecular weight band observed in the non-trasformed plants which indicates for the homotransplastomic nature of plastome. Due to the presence of corresponding band of the low molecular weight observed in the pNAV310-1 is same size to that of non-transformed plant which is an indication of heteroplasmic nature of its plastome (Figure 7.5B). The stable integration of transgenes into plastid genome was further confirmed by reprobing the blots with *co14* coding sequences as probes.

Using the *co14* probe the band on both the transgenic events were detected at about 7.5 kb and no bands on non-transformed genome (Figure 7.5A) and the intensity of the band in pNAV310-6 is stronger than pNAV310-1 indicating higher copy of transgene integration in former than later. Some rearrangement phenomenon might have occurred in pNAV310-1 line as there was slightly higher band than in pNAV310-6 plants. This size difference may be due to rearrangement. The result of the Southern blot using *16S rRNA* probe had confirmed both the genomic DNA samples from transplastomic lines (pNAV310-1 and pNAV310-6) had heteroplasmic integration (Figure 7.5 B) and the hybridazation bands in pNAV310-6 line are slightly lower suggesting the possible rearrangement phenomenon occurring in this line.

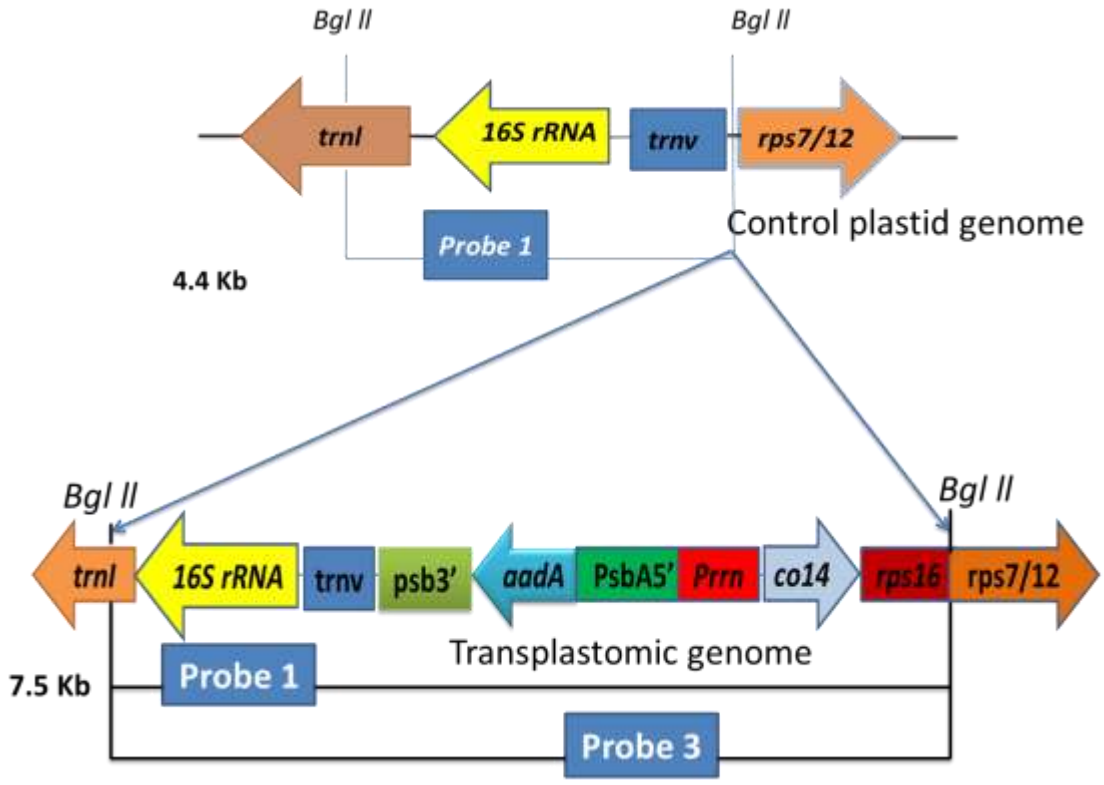
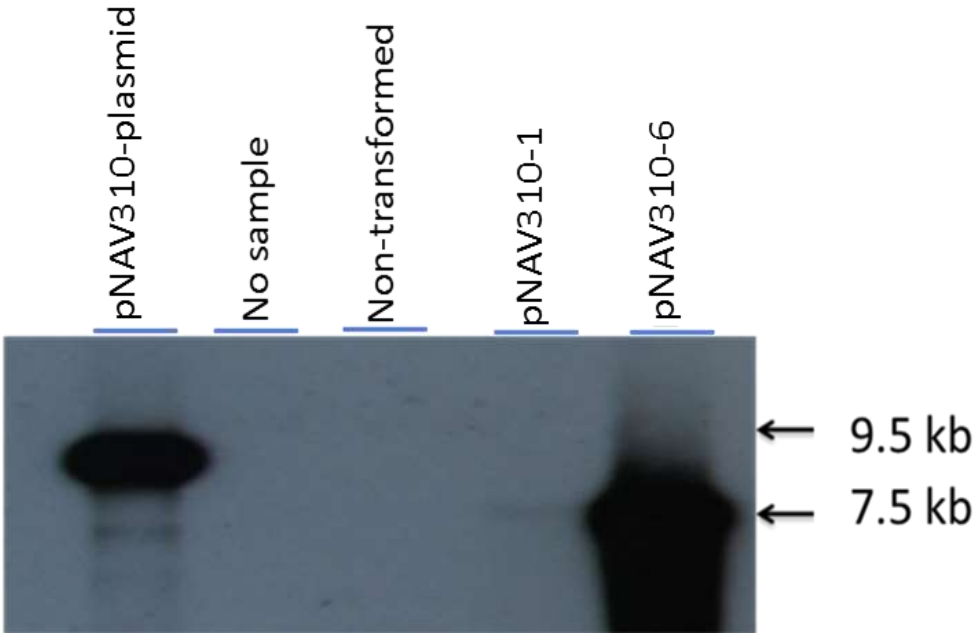
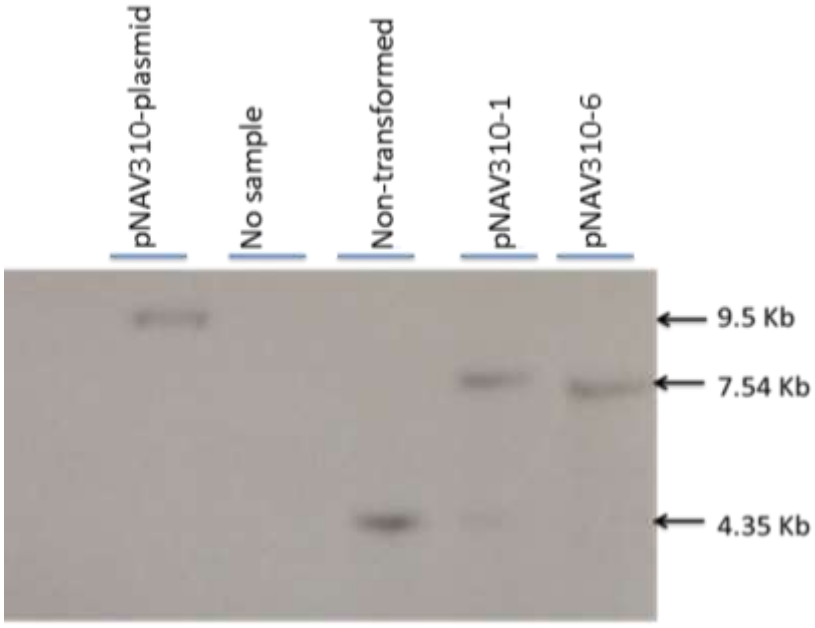


Figure 7.4 Physical map of targeting region of non-transformed (control) plastid genome and the transgenic plastid genome generated with transformation vector pNAV310. The *co14* expression cassette has the ribosomal RNA operon promoter and 3' terminator region of the plastid *rps16*. The selectable marker gene (*aadA*) drives the plastid gene with *PsbA* promoter and *psbA* terminator (Svab and Maliga, 1993).



A.



B.

Figure 7.5 Southern blot analyses for integration of *co14* in transplastomic tobacco plants. Total cellular DNA was digested with *BgI* II and the blot with DNA fragments were hybridised with radiolabelled *co14* and *16S rRNA* probes to detect the genes integration into the plastome genome. Fragment sizes of the non-transformed and the transplastomic lines are indicated as above. As shown (A) represents *co14* probe that investigates integration of expression cassettes and B. *16S rRNA* probes investigate the homoplasmy.

7.4.4 Western blot analysis

For the western blot detection system, *T. reesei* derived polyclonal anti-CBHI antibody was used to detect expression of CO14 from pNAV310-1 and pNAV310-6 plants. The cross reacting band at 54 kDa with the CBHI polyclonal antibody confirmed the presence of the exocellulases (Figure 7.6).

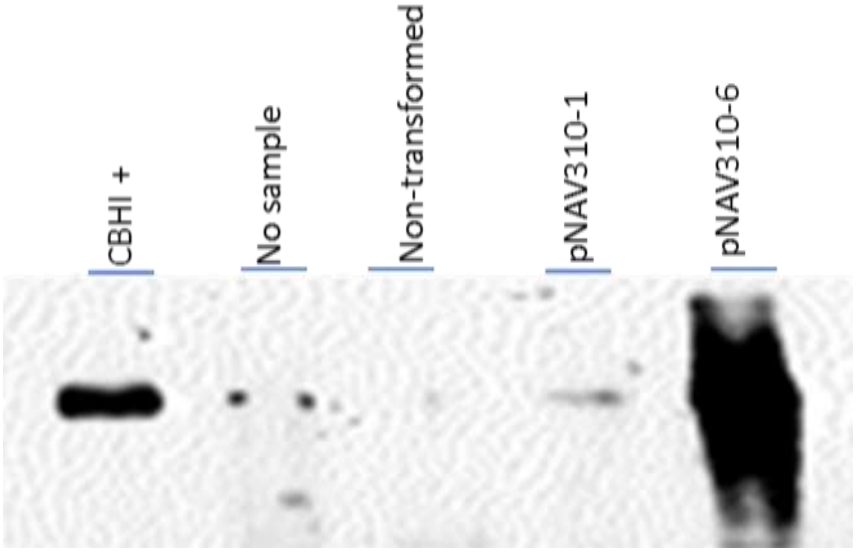


Figure 7.6 Western blot for CO14 accumulation in leaves of pNAV310 plants. Western blot using a polyclonal anti-CBHI antibody against proteins extracted from non-transformed and pNAV310-1 and pNAV310-6 lines. Each lane was loaded with 20 mg of total soluble protein of crushed leaves except for positive control which contain 20 ng of purified CBHI protein.

7.4.5 Enzymatic activity

Although both transformed transgenic lines accumulated CO14, none of the protein extracts had activity against the MUC substrate (data not shown).

7.4.6 Phenotype of of *Prrn-co14* transplastomic plants

7.4.6.1 Phenotypes and chloroplast ultrastructure of *Prrn-co14* transplastomic plants

Both the transplastidic CO14 lines (pNAV310-1 and pNAV310-6) (Figure 7.7) had albino leaves with resistricted plant growth and development. Ultrastructure of chloroplast from transgenic CO14 leaf tissues were again compared with that of non-transformed leaf samples and found that chloroplast structures were altered with considerable rearrangement of the thylakoid membranes (Figure 7.7). There was assembly of plastoglobulins at one corner in chloroplast of pNAV310-1 line and the plastoglobulin are randomy distributed in pNAV310-6 lines. The chloroplast membrane of both the transplastomic lines appears to dissolve. The thylakoid membrane of the pNAV310-6 plants were more severly effected than that of pNAV310-1 plants.

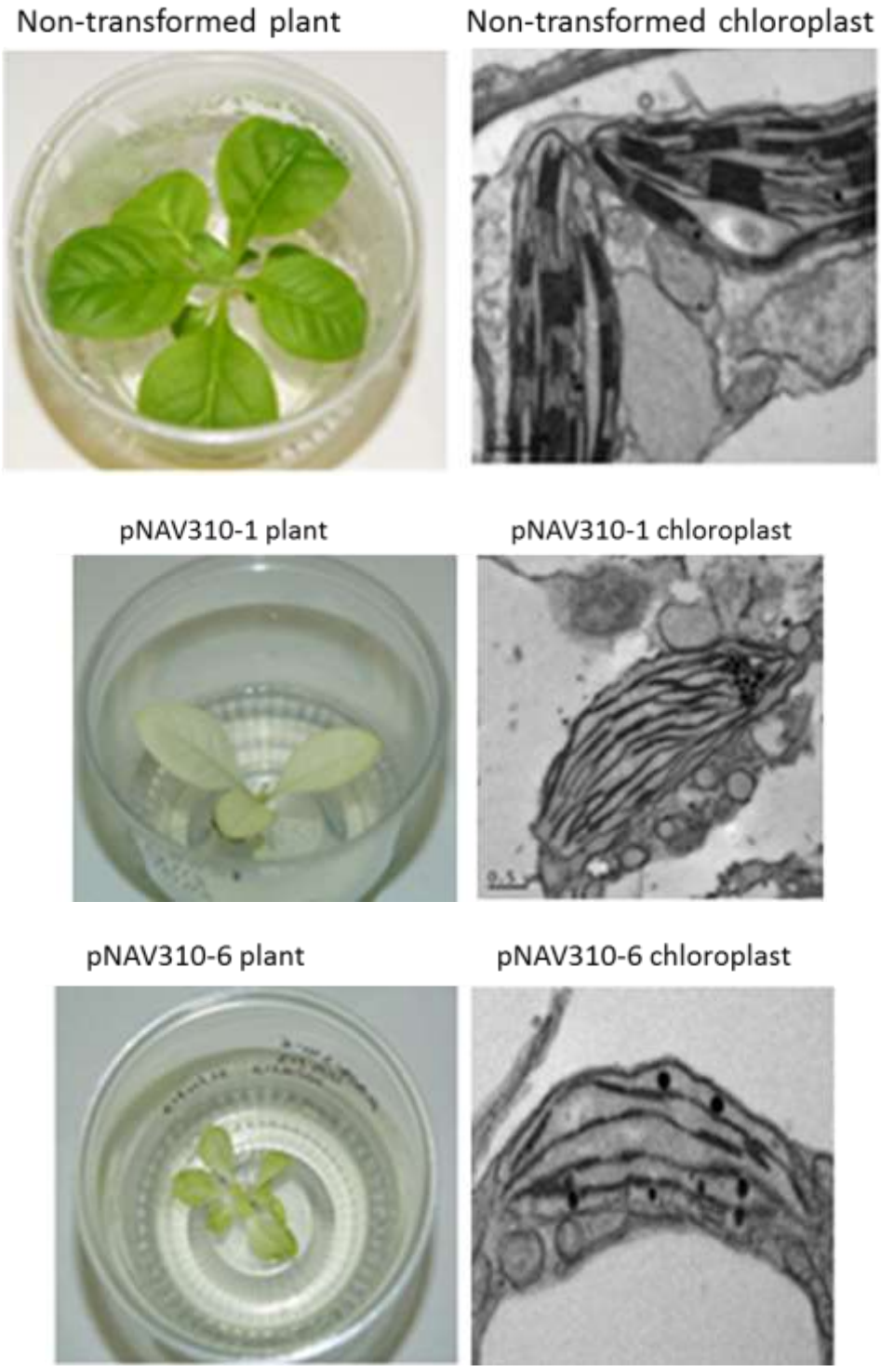
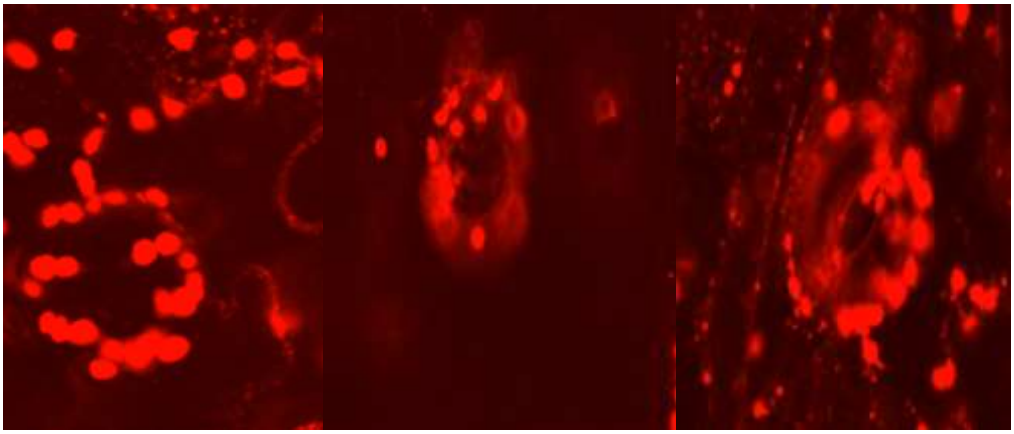


Figure 7.7 Phenotype of non-transformed and plastid transformed CO14 lines. The plant phenotypes were shown parallel with their respective TEM image of chloroplast structures.

7.4.6.2 Guard cell chloroplast visualisation

The chloroplasts count was determined around the guard cells of the both non-transformed and transformed plants using confocal microscopy (Figure 7.8). Both the transplastomic lines (pNAV310-1 and pNAV310-6) had fewer chloroplasts in guard cells compared to the non-transformed chloroplast. The chloroplast number per guard cells were 23 in non-transformed epidermal layer of the leaf, where as the chloroplast count to that of pNAV310-1 and pNAV310-6 plants were 13 and 18 respectively. The chloroplasts in pNAV310-1 and pNAV310-6 plants were distributed randomly while there is uniform distribution of chloroplast in gurd cells of non-transformed plant. Likewise, there were irregular shape of the chloroplast of tansformed lines compared with normal shape and size of the non-transformed plants.



WT pNAV310-1 pNAV310-6

Figure 7.8 Confocal images of the guard cell chloroplasts of the WT versus the transplastidic CO14 lines. Typical chloroplast count around the gurad cell in non-transformed verses pNAV310-1, pNAV310-6 leaves were 23, 13 and 18 respectively, whilst it is not quantitated.

7.5 Discussion

The main aim of this chapter was to generate transplastomic plant expressing *co14* derived from protozoan living in termite gut within the tobacco chloroplast. As outine in the results two c*o14* transplastidic lines (pNAV310-1 and pNAV310-6) out of six spectinomycin resistant shoots were recovered (See section 7.4.1 Regeneration of pNAV310 shoots after bombardment) from 20 bombardments that have been shown to express the recombinant CO14 in TSP of leaves, but the protein activity was not detected.

Both transplastidic plants were selected in MS medium with spectinomycin (500 mg/L) grew very poorly as compared to the plastid transformed GFP plants (Chapter 5). Both the transplastitic lines had an abnormal phenotype characterised by slower growth followed by albino leaf (lacking chlorophyll pigments) with poorly developed roots were observed. This result agreed with the stable transformation of bentgrass with apoplast targeted *co14* resulted in plants with retarded growth and impared root development (personal communication, data not shown by Dr Chung Hong).

At the ultra-structural level, transplastomic chloroplasts had disorganised thylakoid membrane as observed in both pNAV310-1 and pNAV310-6 transplastomic plants. Compared to non-transformed plant, pNAV310-6 lines contained plastoglobules accumulated at one site within the stroma of the chloroplast and in pNAV310-1 plant the plastoglobules were enlarged compared to the non-transformed (Figure 7.7). The exact mechanisms of variation of plastoglobuli size, number and localisation are not completely known. The formation of plastoglobules represents the oxidative stress, senescence and chloroplast-to-chromoplast transition (Austin *et al.,* 2006) and increase in plastoglobules was due to increased the tolerance to light stress (Rey *et*

al., 2000). The possible explanation for larger size of plastoglobules in pNAV310-6 transplastomic plants could be due to imbalance between over-expressed CO14 protein, lipid, enzymes involved in the metabolism of the secondary metabolites there by disturbing the normal functioning of the plant cells. It has been shown that CO14 is detrimental to the plants when expressed in chloroplast, but the actual mechanism of this effect has not been revealed so far.

Chapter 8: General discussion

8.1 Introduction

There has been extensive research on enzyme–mediated conversion of lignocellulosic biomass into simple sugars in order to generate feedstock for ethanol production (Howard *et al.,* 2004, Klein-Marcuschamer *et al.,* 2012). Cellobiohydrolases are recognised as being the most important and rate-limiting enzyme component for the conversion of cellulose into simple sugars. Whilst CBHI is abundantly produced by *Trichoderma*, typically making up to 60% of the total secreted protein (Nummi *et al.,* 1983), improving CBHI expression in filamentous fungi (e. g *T. reesei* or *A. niger*) has been challenging (Zoglowek *et al.,* 2015) and to date production in heterologous expression systems is inadequate to meet the enormous demands required for large scale biofuel production (Park *et al.,* 2015). One approach to overcome this problem is to explore the feasibility of using plants as bioreactors to produce the exocellulases.

The work reported in this thesis sought to explore the use of plants as bioreactors to produce two different exocellulases in large amounts. As mentioned previously, there are challenges in the heterologous expression of cellobiohydrolases and there have been relatively few reports of *in planta* CBHI expression; with only one report of expression in leaves (Dai *et al.,* 1999c) and another of expression in seeds (Hood *et al.,* 2007). The later report involved expression in a plant tissue commonly used for food and feed and large-scale adoption of this approach would clearly present challenges and further debate around resource use for food vs fuel. An extension of leaf expression of cellulases was the main goal of this thesis; the production of exocellulases in tobacco leaves via plastid transformation and expression.

8.2 Summary of the results

At the beginning of this project, two technical aspects that formed the basis of this approach were validated. The method for detection of CBHI; confirming the ability to express and detect the presence of the expression product in tobacco leaves was evaluated via a transient expression system and western blot analysis. Secondly, the plastid expression cassettes designed to express the exocellulases in tobacco chloroplasts were used to express the easily detected GFP protein, thereby confirming the reliability and reproducibility of the plastid transformation system to be used for exocellulase gene expression in tobacco chloroplasts.

Chapter 3 details how *Agrobacterium* harbouring two different *cbhI* expression sequences in plant transformation binary vectors were infiltrated into tobacco leaves and transgene encoded CBHI levels were determined by western blot analysis of the TSP from leaf extracts. Using an anti-CBHI polyclonal antibody a cross reacting band was detected at expected molecular weight, the size being inferred from the coding regions of *cbhI* used in the binary vectors. The antibody clearly cross reacts with purified native CBHI. Estimates of the CBHI levels in the agroinfiltrated tobacco leaves were up to 2.5% of TSP. These levels compare to 17.8% when CBHI is targeted to cell wall and 16.3 TSP % to the endoplasmic reticulum when expressed in seeds of transgenic maize (Hood *et al.,* 2007). Interestingly results in this thesis are 5-fold less than the recently published findings where transient expression of CBHI up to 12.5% of TSP are reported (Hahn *et al.,* 2015). This could be due to fewer number of chloroplast count as the expressed CBHI had some toxic effect that

leads to change of ultrastructure of the chloroplast there by limiting its development and expression rate.

Furthermore, extracts from agroinfiltrated leaves displayed cellulase enzymatic function, as demonstrated in the MUC based assay. Importantly the work reported in this Chapter not only validated the methods used for detection as well as quantitation of plant expressed recombinant CBHI using anti-CBHI antibody; but also validated the enzyme assay used to detect the CBHI activity. Both the methods appeared as reliable and reproducible for the quantitation and detection of CBHI protein and activity in transplastomic plant materials.

Plastid expression cassettes designed to integrate transgene(s) into *16S rRNA* and *rps7/12* region of the chloroplast genome have been reported previously (Maliga, 2002, Maliga, 2004, Daniell *et al.,* 2005). The work reported in Chapter 4 again validates the techniques to be used to examine plastid expression of cellulases. The vectors (with specific *Prrn* promoters and *rps16/psbA* terminator sequences) that were to be used for the plastid cellulase experiments and the bombardment/selection protocols were evaluated. In order to perform these experiments, the coding sequence for the easily scorable and extensively used GFP marker protein were cloned into the various plastid expression cassettes and the expression vectors were used to bombard the tobacco leaves to generate transplastomic GFP plants in this current study.

Whilst there was some variation in the number of transplastidic plants generated per bombardment and levels of the plastid GFP expression between the GFP constructs used, the work generally confirmed the functionality of the plastid expression cassettes and transformation vectors to be used for CBHI expression from the

plastid genome of tobacco. These studies also provided an indication of the general efficiency of the bombardment and selection protocols employed that were to be used to generate CBHI transplastidic plants. The work detailed in Chapter 3 and 4 gave confidence in vectors and protocols to be used for the remainder of the study on *cbhI* plastid transformation expression experiment.

Plastid expression cassettes designed for high level, constitutive expression within the chloroplast often contain promoter regions known to function in prokaryotic host (Maliga, 2002 and Rosales-Mendoza, 2009). Data contained in Chapter 4 show high level GFP expression in plastid expression cassettes with *16S rRNA* promoter and *rps7/12* terminator. As expected, *E. coli* harbouring these vectors also showed expression of the GFP and under UV illumination emit green fluorescence from the expressed GFP protein (data not shown). Therefore, it was expected that expression cassettes containing *cbhI* sequences under the regulation of the *16S rRNA* promoter would also express CBHI protein when maintained in *E. coli* host strains. Interestingly, these cultures when grown at 37^{o}C not only grew slowly, but also showed extensive rearrangement of this expression cassette plasmids (Figure 5.2), which has not previously been reported to be associated with CBHI expression in heterologous systems. As detailed in Chapter 5, significant effort was required to isolate intact/non-rearranged plasmid for bombardment experiments; however, this was obtained by growing *E. coli* at 25^{o}C rather than 37^{o}C for a 24 h growth period. The required quantities of plasmid were obtained from DNA preparations from multiple batches of large volumes of *E. coli* cultures grown at 25^{o}C.

It has been known that *E. coli* was incapable of expression of correctly folded CBHI from *T. reesei*, which was thought to be due to complex arrangement of disulphide

bond in the catalytic domain of the protein (Jeoh *et al.,* 2008). In Chapter 5 results indicate that nascent fungal CBHI protein, when expressed in the *E. coli* host environment appears to not fold correctly; the activity of the CBHI produced from *E. coli* has clearly been compromised as evidenced by the lack of activity in the MUC substrate based assay. The MUC based assay worked very well with purified fungal CBHI. This could be due to effects of differences in pH, osmolarity, redox potential, cofactors, chaperons and folding mechanism in two host systems. Furthermore, it may be that the CBHI expressed in *E. coli* has interacted with metabolites or other intrinsic factors responsible for transcription and translation. The biological function of *E. coli* host cells was clearly compromised displaying slower growth, reduced plasmid copy number and recurrence of plasmid rearrangement phenomenon in the cultures grown at 30^{o}C or 37^{o}C Figure (5.2). However, the plasmid DNA purified from cultures grown at 25^{o}C were found to be intact without rearrangement, thus all the plasmids used for bombardment were obtained from *E. coli* strains maintained and grown at that temperature.

Chapter 6 detailed for the first time the production of transplastomic CBHI tobacco plants. In contrast to the efficient system used to generate transplastomic GFP plants, at a rate of approximately 13 transplastomic per 20 bombardments, only 3 transplastomic *cbhI* transplastidic events were generated from 200 bombardments. No transformed plants were obtained with the pNAV231 vector. Only a single event was generated from each of the 3 plasmids; pNAV230, pNAV232 and pNAV233.

The PCR and Southern blot analysis confirmed the integration of the *cbhI* expression cassettes into the chloroplast genome of the transplastomics that were obtained. Both the PCR and Southern blot showed the band of the expected size in pNAV230

and pNAV233 generated lines, whereas there were additional fragments inserted within the *cbhI* region in pNAV232 line (Figure 6.3 and 6.5). Given the plasmid DNA used for the bombardments was checked for integrity by PCR and appeared to be intact and not rearranged; it would seem unlikely that the expression cassettes were rearranged prior to the bombardment. What may be more likely is that the rearrangement occurred after or during the integration of the *cbhI* expression cassettes into the chloroplast genome. This phenomenon of rearrangement of foreign gene sequences during or after integration within the inverted sequences in the chloroplast genome have been reported previously (Gray *et al.,* 2009) and it may be that the *cbhI* cassettes are for some reason more prone to rearrangement, resulting in the changes observed in the pNAV232 transplastomic line.

Using an anti- CBHI antibody for western blot analysis recombinant CBHI was detected in the TSP extracts of pNAV230 and pNAV233 transplastomic lines. The levels of CBHI accumulation in the pNAV230 lines were 0.5% of TSP and in the pNAV233 line was 0.02% of TSP. However, no CBHI was detected in the pNAV232 plant, even though, there appeared to be some detrimental biological effects of CBHI, as evidenced by the disrupted chloroplasts and fewer numbers of chloroplasts in pNAV232 lines compared with the control GFP trasplastomic plants (Figure 6.9). However, no CBHI activity was detected from TSP isolated from of either of the CBHI transplastomic lines when analysis was performed with MUC substrate base assay. This could be also due to complex structure of fungal CBHI, perhaps that chloroplast environment does not necessarily provide an environment or chaperone systems that is able to fold the fungal CBHI protein correctly or in a form that can cleave the MUC substrate in the *in vitro* assay.

The CBHI transplastomic plants generated in this study have clear pleiotropic effects; including beaching of leaves, slow growth and the plants could not survive when transferred into soil. The regeneration/rooting time of transplastomic CBHI expressing transplastomic lines were up to 320 days as compared to the normal rooting time of 60-90 days observed for the non-transformed plants or transplastomic GFP lines (data not shown). This difference in regeneration or rooting time, pale leaves, weaker roots and slower growth was also reported for transplastomic lines expressing XynA-CEC3 line (Kolotilin *et al.,* 2013) and swollenin (Verma *et al.,* 2013), indicating some level of consistency with data in this study with other published studies.

Interestingly, the ultrastruture of chloroplast from both the transplastomic CBHI in this study (Figure 6.9) and transplastomic swollenin expressing plants displayed similar alterations (Verma *et al.,* 2013). Plastid expressing CBHI and swollenin plants both show a characteristic reduction in chloroplast volume, unstacking and degradation of thylakoid membranes, immature thylakoid system and increase in plastoglobulin. The chloroplasts from both the swollenin and CBHI transplastomic plants were found to be globular, elongated and rectangular in structure; and in some cases, had plastoglobuli that could be associated with disjointed chloroplast outer-membranes. Unfortunately no ultrastructure data for xylanase expressing plants was reported (Kolotilin *et al.,* 2013). As mentioned previously in Chapter 6 transplastomic plants expressing CBHI lacked activity on MUC substrate, their ultrastructure has a similar degree of chloroplast disruption compared with swollenin transplastomic plant chloroplast which were however functionally active and showed expansion activity in cotton fibre, resulting in irreversible unwinding of cellulose micro-fibril (Verma *et al.,* 2013).

A surprising observation in this study was that even though pNAV230 transplastomic plant has 10-fold more CBHI than pNAV233, the observed detrimental phenotype seems less severe than that observed in the pNAV233 lines. This phenotypic difference observed in CBHI transplastomic lines could be related to the design of the expression cassettes (Table 6.1). It was expected that presence of enhancer sequence (k) in pNAV232 and pNAV233 vectors would have enhanced expression of CBHI consistent with data for GFP expression where pNAV207 plant have enriched expression when compared with pNAV205 (Figure 4.8 and 4.8). The accumulation of CBHI protein from each of the two cassettes in transplastomic tobacco plants correlated with RNA levels for the *cbhI* gene (Figure 6.7 and 6.8). The pNAV230 plants showed higher *cbhI* RNA and CBHI protein, while accumulation of CBHI expressed from pNAV233 was lower and its *cbhI* RNA band was not as stronger as that in pNAV230 plants. This could be due to the difference of the total number of chloroplasts in pNAV230 and pNAV233 plants; for example, chloroplast count of pNAV233 plant was about 10^4 times less than that of pNAV230 plant (data not shown) and this difference in the number of chloroplast is consistent with the difference in the leaf phenotype of the two transplastomic plants (Figure 6.1). Thus the western blot data (Figure 6.7 A) did not pick up the CBHI band from the pNAV233 plant TSP extracts, as there were fewer chloroplast number compared to that of pNAV230 plants, but when enriched chloroplast TSP from pNAV233 plant was examined and CBHI protein was detected (Figure 6.7B). These studies conclude that the highest level of expression of CBHI in pNAV233 plants resulted in more severe effects on the chloroplast and maybe related to the previously proposed theory of disruption of plastid mRNA homeostasis by massively redirecting mRNA synthesis there by causing reduced transcription of essential genes and limiting

genetic materials within the transformed chloroplast (Petersen and Bock, 2011). These type of effects have been reported by previously by authors in the transplastomic plant expressing cell wall degrading enzymes (Petersen and Bock, 2011), xylanase (Kolotilin *et al.,* 2013) and swollenin (Verma *et al.,* 2013).

There are several multi-subunit protein complexes within the chloroplast; photosystem I (PSI), photosystem II (PSII), light-harvesting complex-I (LHC-I), LHC-II, cytochrome b6/f complex and ATP synthase embedded within the chloroplast membrane system (Dekker and Boekema, 2005, Jensen *et al.,* 2007), but the functionality or levels of these complexes were not examined in CBHI expressing transplastomics, however, this would be worth examination. In swollenin expressing transplastidic plants it was found that there was reduction in the levels of monogalactosyldiacylglycerol (MGDG) and diagalactosyldiacylglycerol (DGDG); however, the estimation of level of these molecules was not conducted in the current project but are worthy of investigation. The disruption of the internal structures of chloroplasts observed in transplastomic CBHI plants could possibly be related to destabilisation of these photosynthetic protein complexes of the thylakoid membrane, which would in turn have the adverse effects on the plant phenotype. Future studies on level and function of some of these photosynthetic protein complexes may help understand how CBHI seems to be altering the plant phenotype so markedly.

Chapter 7 detailed for the first time the production of CO14 from termite gut protozoan in transplastomic tobacco plants. Again, in contrast to the efficient generation of transplastomic GFP plants, only 2 transplastomic CO14 events were generated from 20 bombardments.

Southern blot data (Figure 7.5) confirmed the integration of the *co14* expression cassettes into the chloroplast genome of transplastomic tobacco plants. The pNAV310-1 plant was heteroplasmic however; the pNAV310-6 plant appeared to have homosplasmic integration. The size of larger hybridising fragment observed in the Southern blot of pNAV310-6 plant DNA was slightly lower compared to that of pNAV310-1 fragment (Figure 7.5). This again could be the due to a rearrangement phenomenon. The rearrangement phenomenon was also observed in the pNAV232 plant (Figure 6.5) which in contrast shows that there was addition of extra sequence within the *cbhI* fragment (Figure 6.3). The observed phenotype of the pNAV310-1 plant seems to be mild with effects on the chloroplast and the slightly broader leaves as compared to the severe disruption of the chloroplast structure in pNAV310-6 plant phenotype (Figure 7.7), while the pNAV232 plant had altered chloroplast and the plant could not differentiate into proper roots, stems and leaves.

The inferred structure of CO14 (a putative 1Q9H from *Talaramyces emersonii)* was superimposed with the complete structure of *T. reesei* Cel7A (Voutilainen, et L., 2010), by Dr Chung Hong, (personal communication, data not shown) and was also found to have some regions of strong homology with Cel7A. It was also found that an anti-CBHI antibody could cross react with CO14 proteins. This same antibody was subsequently used for detection and approximate quantitation of CO14 in the TSP extracts of pNAV310-1 and pNAV310-6 transplastomic lines. As expected there was 10-20-fold increase in the levels of the CO14 observed in pNAV310-6 than that observed in pNAV310-1 plants. Whilst there was no activity of C014 as determined by the MUC substrate assay, however, expression in the plant did appear to have a biological effect and the transformed chloroplast structures been altered drastically, pNAV310-6 plants had severely distorted ultrastructure compared to pNAV310-1

(Figure 7.5 and 7.7). This data is reminiscent of the previous results observed for CBHI expressing plants (Figure 6.1 and 6.5).

Thus, the work reported in this thesis indicates that the expression of either CBHI from *T. reesei* or CO14 derived from protozoan living in the termite gut in the chloroplast of tobacco have similar disruptive effects on chloroplast function and ultrastructure. These results were consistent with previously reported expression and accumulation of cell wall degrading enzymes in chloroplast and have major impacts on transplastomic plants (Kolotillin *et al.,* 2013).

Hence this thesis concludes that expression of either exocellulase CBHI or the putative exocellulaseCO14) under the regulation of the constitutive *Prrn* promoter in tobacco chloroplasts is technically possible as has been deomonstrated by the generation of transplastidic plants, however, there were significant detrimental effects on chloroplasts growth and plant development. Whilst successful in the generation of transplastomic plant expressing CBHI/CO14, these observations indicate there are significant challenges in regeneration of plants and expression of these full-length CBHI/CO14 proteins in chloroplasts, which may suggest that chloroplast, is not suitable organelle for targeted expression of the exocellulases.

The amounts of CBHI produced in transplastidic plants were significantly lower than the amount required for hydrolysis of the biomass. It is important to consider all factors concerning the expression levels and in particular the types of celluases to be expressed in relation to localization within the plant. It is interesting to note that work in our laboratory indicates that the expression of the bacterial E1 endocellulase in transplastic tobacco using exactly the same expression cassettes was able to achieve levels of E1 protein up to 50% of TSP without the significant phenotypic

alterations observed for CBHI or CO14 protein expression. This E1 protein also displayed substantial MUC substrate based activity (Phillip Dix, unpublished data). In contrast, CBHI/CO14 expressed using the same expression cassettes in the same tobacco plant lead to severe detrimental plant phenotypes and altered chloroplast structures and non-functional enzyme. As a result, the heterologous expression of individual cellulases should be examined independently for the effects on plant growth and development, as well as for enzyme yield and activity. Despite this, plant systems remain one of the most promising methods for cellulase production on a large and sustainable scale.

8.3 Conclusion

The proposed aim of this project was to produce *in planta* expression of exocellulases gene in *N. tobacum*. In this study, CBHI from *T. reesei* and exocellulases (CO14) from termite gut protist were expressed in tobacco chloroplasts. Homoplasmy was achieved in one of the CO14 transplastomic line (pNAV310-6). While the pNAV310-1 line and pNAV230, pNAV232 and pNAV233 lines were heteroplasmic in nature. Expressed CBHI and CO14 in chloroplast caused drastic changes in chloroplasts structure and plant phenotype.

It appears likely that lower level enzyme accumulation might be due to the limiting factor of the transcription and translation machinery within the chloroplast and this type of deleterious effect have been observed previously in other transgene expression studies (Tregoning *et al.,* 2003). One of the interesting finding of this project was that expression of exocellulases both (CBHI and CO14) affected the

growth and development. These results were consistent with previously reported expression and accumulation of cell wall degrading enzymes in chloroplast that have major impacts on phenotype of the transplastomic plants (Petersen and Bock, 2011).

The overall conclusion of this thesis is that the chloroplast is not a suitable organelle for the expression of the exocellulases (CBHI/CO14). There was significant rearrangement of chloroplast genome, an absence of cellulolytic activity of expressed protein and disorganisation of the thylakoid membrane in plants expressing these enzymes in the chloroplast. Extension of this approach to biomass crops for biofuel production still presents major technical and biological challenges. The use of inducible expression for production-scale application remains a future challenge for plastid transformation.

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Sequence of the plasmids CBHI vectors: CBHI full length: 1588bp

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cggatattctggacctactgtatgtgcttctggaactacttgtcaggtattgaatccttattattctcaatgtctttga
attcggcgcgccctgggcctcatgggccttcctttcactgcc

Amino acid sequence CBHI full length 528 amino acids

From (ORF Finder (Open Reading Frame Finder): sequence1 frame +1

PWLVLNLLVLCNLKLILL*LGKNVLLVELVLNKLDL**LMLIGVGLMLLILLLIVMM
EILGVLLCVLIMKLVLKIVVWMVLLMLLLMV*LLLEILCLLDS*LNLLKKM*VLVCI
*WLLILLIKNLLCWEMNFLSM*MYLNCLVD*MVLCISYLWMLMVEYLNILLILLV
LNMELDIVILNVLVI*NSLMDKLM*KDGNLLLIMLILELEDMDLVVLKWIFGKLIL
FLKLLLLILVLL*DKKFVKVMDVEELILIIVMVVLVILMDVIGILIVWEILLSMDLDL
LSLWILLKN*L**LNSKLLVLLIVIMYKMV*LSNNLMLNWDLILEMN*MMIIVLLKK
LNLEVLLSLIKVD*LNSKKLLLVVWYW*CLCGMIIMLICCGWILLIQQMKLLLLL
VL*EDLVLLLLVFLLK*NLNLLMLK*LSLILNSDLLDLLVTLLVEIKN.

CBHI cat sequence: 1331 bp

ccatggctcgtgctcaatctgcttgtactttgcaatctgaaactcatcctcctttgacttggcaaaaatgttcttctg
gtggaacttgtactcaacaaactggatctgtagtaattgatgctaattggcgttggactcatgctactaattcttct
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aatctcctaatgctaaagtaactttctctaatattaaattcggacctattggatctactggtaacccttctggtgga
aattaagaattc

from ORF Finder (Open Reading Frame Finder) Sequence 1 fram +1

PWLVLNLLVLCNLKLILL*LGKNVLLVELVLNKLDL**LMLIGVGLMLLILLLIVMM
EILGVLLCVLIMKLVLKIVVWMVLLMLLLMV*LLLEILCLLDS*LNLLKKM*VLVCI
*WLLILLIKNLLCWEMNFLSM*MYLNCLVD*MVLCISYLWMLMVEYLNILLILLV
LNMELDIVILNVLVI*NSLMDKLM*KDGNLLLIMLILELEDMDLVVLKWIFGKLIL
FLKLLLLILVLL*DKKFVKVMDVEELILIIVMVVLVILMDVIGILIVWEILLSMDLDL
LSLWILLKN*L**LNSKLLVLLIVIMYKMV*LSNNLMLNWDLILEMN*MMIIVLLKK
LNLEVLLSLIKVD*LNSKKLLLVVWYW*CLCGMIIMLICCGWILLIQQMKLLLLL
VL*EDLVLLLLVFLLK*NLNLLMLK*LSLILNSDLLDLLVTLLVEIKN

Appendix 1 – Solution and media

Agarose gel Electrophoresis Gel

DNA Grade Agarose 1% (W/V)

Made up with 1X TAE Buffer

Antibiotics stock Solution:
Amphicillin 300 mg/mL in H_2O
Gentamycin 100 mg/mL in H_2O
Kanamycin 100 mg/mL in H_2O
Spectinomycin 500 mg/mL in H_2O

Bromphenol Blue Dye (6X)

Bromphenol Blue	0.25%
Glycerol	30%
Xylene Cyanol	0.25%

MS liquid Medium

MS salts Mixture with (Invitrogen)	4.43 g/L
Sucrose	30g/l
Adjust pH	5.8
Phytogel	4g/L

MS Regeneration Medium:

MS liquid medium

Benzyl-amino-purine (BAP)	1 mg/mL
Napthalene-acetic acid	0.1 mg/mL

MS Regeneration Medium + selection Medium:
MS regeneration Medium + 500 mg/L Spectinomycin

MS selection medium:
MS medium + 500 mg/L spectinomycin